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APPROVAL SHEET

Title of Thesis: Characterization of the Polypeptides in Varicella Zoster Virus - Infected Cells

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Doctor of Philosophy Degree
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ABSTRACT

Title of Dissertation: Characterization of the Proteins in Varicella-Zoster Infected Cells.

Chester Ronald Roberts, Doctor of Philosophy, 1984

Dissertation directed by: John Hay, PhD
Professor of Microbiology
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The structural and functional proteins expressed by Varicella-Zoster virus (VZV), the agent that causes chickenpox and shingles, were examined at the infected cell level and the virion level. To accomplish this a new cell line of human foreskin fibroblasts was employed for the growth of the virus. In addition a unique virion isolation technique was developed using sucrose zonal centrifugation followed by a combination of equilibrium-viscosity sedimentation with potassium tartrate-glycerol gradients. This resulted in the identification of 39 infected cell polypeptides ranging in molecular weight from 240K to 21K. Twenty-nine structural proteins of virions and nucleocapsids were also identified with molecular weights ranging from 175K to 21K. Using various radiolabels the phosphoproteins and glycoproteins of VZV were also identified. The 175K polypeptide was identified as the major phosphoprotein and was found only in virions and not in nucleocapsids. It is suggested that this is a major "tegument" protein of VZV. The 155K protein was identified as the major capsid protein of VZV and an ultrastructural comparison with the major capsid protein of HSV-2 was made

using disrupted nucleocapsids.

Functional studies on the polypeptides of VZV were done by DNA cellulose chromatography and protein blotting to nitrocellulose filters in order to characterize the DNA binding proteins. Seven major DNA binding proteins were identified with molecular weights ranging from 175K to 21K. The 125K DNA binding protein was identified as the major DNA binding protein of VZV. Ten to twelve phosphoproteins were also identified as DNA binding and had molecular weights ranging from 175K to 34K. Differences in affinity for DNA were noted. The protein blot procedure was also used to identify DNA binding proteins and the specificity of binding between homologous, nonhomologous, and "Z" DNAs was studied. In addition to the DNA binding proteins, other proteins were identified as "immediate early" using cycloheximide block and release techniques. Four such "immediate early" polypeptides were identified with molecular weights of 175-180K, 100K, 60K, and 28K. Three stress proteins that appear to be stimulated by VZV infection were noted at 92K, 81K, and 68K. Using the Western blot technique the 155K major capsid protein was shown to cross-react with HSV-1 antisera indicating shared antigenic determinants with HSV-1. Attempts were made to map the thymidine kinase gene of VZV using dot blot hybridization and transfection techniques. Although conclusive proof is lacking, it is believed that the gene is located close to the EcoRI D-L junction.

Since we have identified the proteins of VZV nucleocapsids and compared them to those of HSV-2, it seemed of interest to investigate similarities and differences in the nucleocapsid structures of these two viruses using computer assisted high resolution electron microscopy. Using a number of techniques, we have been able to generate a

sufficient number of high quality electron micrographs to show the nucleocapsid structure of VZV and HSV-2. In the near future this data will be subject to computer analysis and high resolution comparisons will be made.

CHARACTERIZATION OF THE PROTEINS

IN

VARICELLA-ZOSTER VIRUS INFECTED CELLS

BY

CHESTER RONALD ROBERTS

Thesis submitted to the Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services University of
the Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy, 1984

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"No man is an island." I owe thanks to so many for the encouragement, direction, and help that made this work possible. To list them invites inadvertent omission and therefore hurt feelings. From my family to my scientific colleagues and personal friends, all played important parts in this production. You know who you are and I hope you also know that I will be forever grateful. I hereby dedicate this work to all of you.

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INTRODUCTION

Viruses belonging to the Herpetoviridae family are today having a tremendous impact on mankind, not only medically and scientifically, but socially as well. Varicella-Zoster virus is one of the five major members of the herpesviruses that infect man, the others being herpes simplex type 1 (HSV-1), herpes simplex type 2 (HSV-2), cytomegalovirus (CMV), and Epstein-Barr (EBV) virus. Varicella-Zoster virus (VZV) is currently the cause of the third most common complaint that a physician encounters in his practice (Steel, 1982). The primary infection is seen mainly in children and is known in the vernacular as "chickenpox". In adults this childhood infection resurrects itself as a painful condition known as "shingles".

Studies on the origin of the term chickenpox have revealed conflicting interpretations. Steele (1982) reports that chickenpox is from the Latin cicer, meaning chick pea, a common plant of Europe whose seeds look like the lesions of chickenpox. Scott-Wilson (1978), on the other hand, suggests that the origin is from the Old English "gican" meaning to itch. He reports that the first use of the word chickenpox was in 1694 describing the only disease prevalent at that time which, when it appeared, itched and for the most part went away without problems and so set it apart from other pox-like diseases that caused death or disfigurement. These early observations also gave rise to the term varicella, from the Latin varius, meaning mottled or various, since it was a disease which demonstrated lesions like smallpox, albeit of a different kind.

The term "shingles" was used as early as the fifth century B.C. by Hippocrates and comes from the Latin singulus, meaning girdle, referring

to the fact that the skin lesions spread around the chest like a girdle (Taylor-Robinson and Caunt,1972; Gold and Nankervis, 1973). The term zoster, which is derived from the Greek word for girdle, also refers to this common clinical manifestation of the disease. Because the lesions spread, the term "herpes", from the Greek word meaning to creep, was also applied to this and other lesions of the skin irrespective of etiology. Currently most refer to the primary disease as chickenpox and to the recrudescence as zoster or shingles.

Although recognized as two different diseases, the idea that the same etiological agent caused both chickenpox and zoster was first suggested by Von Bokay (1909) when he reported the occurrence of chickenpox in families who had just previously had a case of shingles. In partial fulfillment of Koch's postulates, Kundratitz in 1925 produced chickenpox with vesicle fluid from a patient with zoster. The viral agent was first isolated in tissue culture by Weller in 1953 and the door was finally open for laboratory investigation into the molecular biology of the virus.

When first clearly viewed under the electron microscope (Tournier et al., 1957; Long et al., 1970), Varicella-Zoster virus looked structurally identical to a well known pair of viruses, HSV-1 and HSV-2. It was thus classified in the Herpetoviridae family. The Herpetoviridae family, in addition to VZV, now contains approximately 70 different viruses with a host range from fungi to primates (Brunell, 1979; Spear and Roizman, 1980). VZV meets many of the criteria established by Andrews (1964) for inclusion into the Herpetoviridae. The virus is composed of four major parts: the envelope, the tegument, the capsid, and the core. The capsid is icosahedral with 162 capsomeres

and the whole virion varies in size from 100 to 150 nm in diameter. The core contains DNA presumably complexed with protein and organized in an undefined manner (Ludwig et al., 1972). The nucleocapsid structure is assembled in the nuclei of infected cells and buds through the nuclear membrane where it appears to acquire part of its envelope (Hay and Watson, 1983). The rest of the journey to the outside of the cell is still not clear and VZV has not been clearly shown to receive an envelope from any cytoplasmic structure. This may be a reflection of the fact that VZV has long been known to be strongly cell associated (Weller, 1953; Weller, 1983). Other characteristics of the Herpetoviridae family, and thus VZV, include the lack of hemagglutinin, no antigen common to the whole group but some crossreactivity amongst certain viruses of the group, production of lesions which become necrotic, intranuclear inclusions of Cowdry's type A, and, in some instances, involvement of the central nervous system (McCormick et al. 1969; Taylor-Robinson and Caunt 1972; Ghatak and Zimmerman, 1973; Wildy, 1973; Bastian et al., 1974).

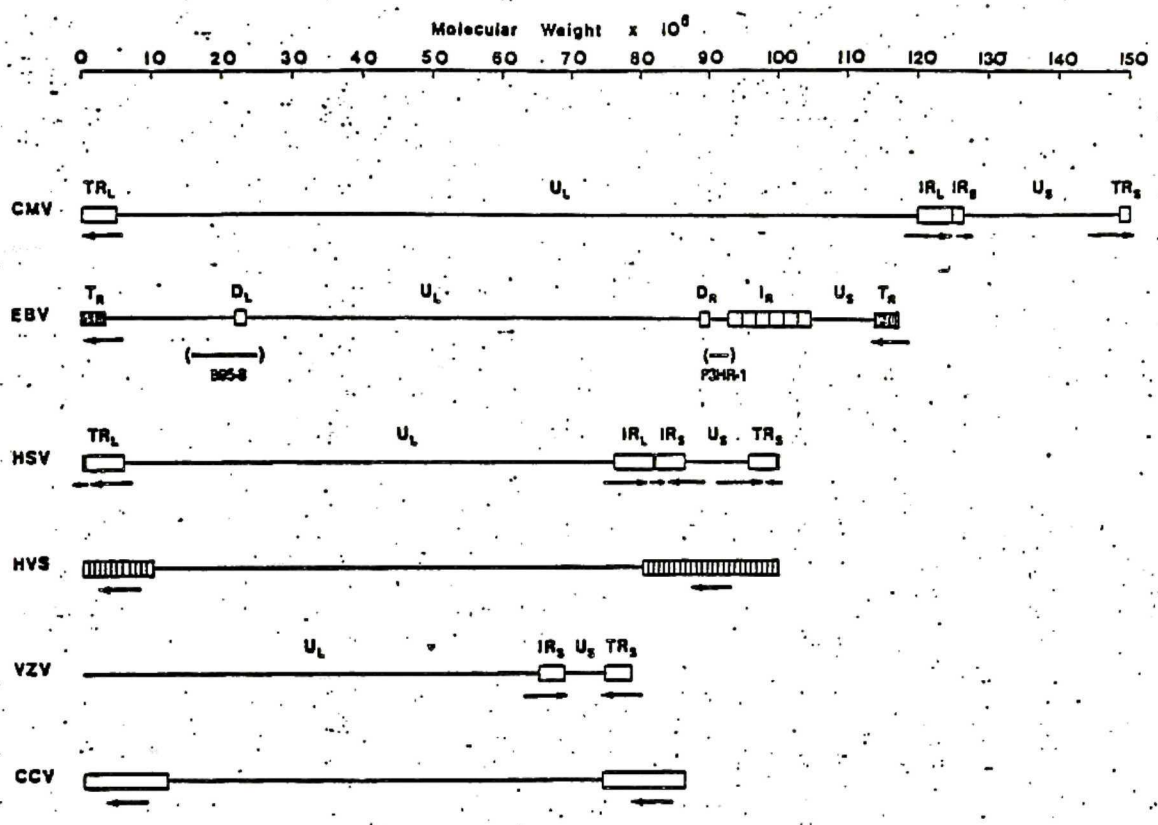
It is curious that VZV is so highly contagious in human populations and yet is so difficult to handle in tissue culture systems. Since cell free infectious virus titers are very low, the studies possible with the herpes simplex viruses have not been applied to VZV. As a result the molecular biology of VZV has lagged far behind that of HSV-1 and HSV-2. What little is known has shown that VZV is different in many respects from HSV and that any comparisons of the molecular biology between VZV and HSV must be made carefully. In order to avoid confusion, the molecular biology of VZV will be reviewed first followed by an overview of HSV.

VZV DNA.

Recent studies have estimated that the molecular weight of VZV DNA is 72-80 x 10^6 daltons (Straus et al ., 1981; Ecker and Hyman, 1982). Figure 1 compares the genomes of members of the herpesvirus family and demonstrates the similarities as well as the differences within the family. As can be seen VZV consists of a short unique region (US) flanked by inverted repeats (IR) followed by a unique long region (UL). It has also been suggested that there is a small (40 base pair) inverted repeat region at the ends of the genome (Davidson, 1983). Restriction endonuclease digestion studies have shown small differences between various strains of VZV. These differences were seen mainly in the right terminal inverted repeats and in the middle of the unique long region (Straus et al.,1983; Hay and Watson, 1983). The G+C content is 46-48% which is much lower than that of the herpes simplex viruses and results in the VZV DNA copurifying with cellular DNA on CsCl density gradients. This combined with the strong cell association of the virus has made purification of large amounts of viral DNA difficult. To partially surmount this problem, restriction endonuclease fragments of VZV were cloned into various vectors and enough DNA was isolated to perform hybridization and sequencing experiments (Straus et al.,1982; Davidson and Wilkie; 1983, Davidson, 1983). Preliminary results have indicated some homology between HSV-1 and VZV DNA. Sequencing data is just beginning to be published and suggests a gene order similar to HSV-1 (Davidson and Wilkie, 1983; Davidson, 1983; Davidson, in press).

VZV Proteins

As a result of its cell-associated nature, VZ virion purification



Comparison of the DNA structures of human cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), *Herpesvirus saimiri* (HVS), varicella zoster virus (VZV), and channel catfish virus (CCV). Unique sequences are represented by a single line, while the reiterated sequences are represented by open boxes. Arrows indicate the relative orientation of the repeated sequences. Sequences deleted in EBV strains B95-8 and P3HR-1 are shown bounded in parentheses.

Figure 1. Reproduced from Hay and Watson (1983).

and analysis of VZV-specific proteins has proven difficult. For example, it is not possible to obtain high titered cell free virus with current culture systems and so infected cells have for the most part been used to initiate infections. As many as two billion cell-free virus particles per ml of media have been counted with no detectable infectivity (Shiraki and Takahashi, 1982). It has been suggested that the envelope of VZV is extremely labile to enzymes or physical damage when grown in tissue culture (Cook and Stevens, 1968). VZV also does not turn off host-cell protein synthesis in the dramatic way seen with HSV and so host cell background must be taken into consideration. It is obvious, therefore, that a synchronous infection to detect uniformly labeled virus specific proteins is difficult to achieve.

All these problems with VZV are reflected by the fact that only fourteen papers exist in the literature on the proteins of VZV since it was first grown in tissue culture in 1953. The first published report on the polypeptides of VZ was by Wolff (1978) who reported a pattern of 31 proteins ranging in molecular weight from 240K to 18K of which 14 were immunoprecipitated with human convalescent antisera. This study used radiolabeled virions partially purified on a sucrose density gradient (10-60% w/v). There were no comparisons with uninfected cell polypeptides. Shemer et al.(1980) reported on an improved viral isolation technique that employed zone centrifugation as a first step in purification followed by a combination equilibrium - viscosity gradient sedimentation with potassium tartrate-glycerol (KT/GLY) gradients (30-50% w/w). The degree of purification was seventy-fold with respect to host proteins and the ^{35}S-methionine or ^{14}C-glucosamine labeled virions were subjected to SDS-polyacrylamide gel electro-

Table I Comparison of VZV Polypeptides Cited in the Literature*

Wolff		Shemer	Takahashi	Zweerink	Grose
(IP)				(IP)	(IP)
240		244	280	200+	200+
149	149	222	220	155	174
142		180	180	130	145
140		153	155	88	126
110	110	141	145	73	118
108	108	120	130	67	98
96		100	118	60	88
89		80	115	57	76
85		77	100	39	62
73	73	72.5	89	37	52
70	70	70.5	86	34.5	48
65		67	80	33	45
61		65	71	31.5	38
55		63	64	22.5	37
53	53	60	59	17.5	34
52		58	57		
48		57	55		
44		55	54		
41		52	51		
39	39	49.5	48		
37	38	47	45		
35	32	45.5	42		
31	30	43.5	38		
28	28	42	35		
27		38	33.5		
25		36	32		
23	23	34.5	30		
21	21	32	28		
19		29.5	25		
18		28	23.5		
		19.25	21		
		16.75			
		16			

*Compiled from: Wolff (1978), Shemer et al. (1980), Asano and Takahasi (1980), Zweerink and Neff (1981), Grose and Friedricks (1982), Takahashi (1983).

phoresis. They found 33 species of polypeptides with molecular weights ranging from 244K to 16K and 5 glycoproteins ranging in molecular weight from 140K to 52K. It was also suggested that the major component of VZV nucleocapsids had a molecular weight of 180K. Shiraki et al. (1982, from Takahashi's group at Osaka University, Japan) reported on a three-step procedure involving sucrose density gradients (10-15%) of a cytoplasmic extract followed by equilibrium centrifugation in CsCl. The degree of purification reported was 85 fold with respect to host proteins in an experiment with an artificial mixture of infected and uninfected cells. By this technique 32 polypeptides ranging in molecular weight from 280K to 21.5K were found in the virion. This included 6 glycoproteins ranging in molecular weight from 115K to 45K. The major polypeptide of VZV nucleocapsids was reported to have a molecular weight of 145K.

Two other groups, Zweerink and Neff (1981) and Grose and Friedricks (1982) have also reported on VZV polypeptides. They, along with Wolff (1978) used immunoprecipitates to isolate the viral polypeptides and reported only 14 to 15 polypeptides with a range of 200K to 17.5K in molecular weight. The low number of polypeptides isolated is no doubt due to the polyclonal antibodies used in these studies.

Table I summarizes the VZV polypeptide molecular weights reported to date. As can be seen, there are significant differences in the literature between assessments of the polypeptide composition of VZV.

VZV Glycoproteins

The study of VZV glycoproteins has also suffered because of the cell-associated nature of the virus. However, monoclonal antibodies against some of the major glycoproteins have been developed and used in

TABLE II

COMPARISON AND TENTATIVE ARRANGEMENT OF VZV-SPECIFIC GLYCOPEPTIDES CITED IN THE LITERATURE

Source of glycopeptide	Molecular weight (×1000)	Reference
Infected cells	118 (major)	Grose (1980)
	98 (major)	
	88	
	62 (major)	
	45	
Partially purified virion	118	Grose (1980)
	98	
	62	
Purified virion	141, 120 } gp1 (major)	Shemer *et al.* (1980)
	100, 80 } gp2 (major)	
Purified virion (*continued*)	67: gp3	Shemer *et al.* (1980)
	63: gp4 (major)	
	52: gp5	
Infected cells	130	Zweerink and Neff (1981)
	88	
	60	
Purified virion	115: gp1 (major)	Shiraki *et al.* (1982)
	100, 80 } gp2 (major)	
	64: gp3 (major)	
	59: gp4	
	55: gp5 (major)	
	45: gp6	

Table 2. Reproduced from Takahashi (1983).

immunoprecipitation experiments (Janot et al.; Grose et al., 1983; Okuno et al., 1983). A summary of these glycoprotein studies is shown in Table 2 (Takahashi,1983). A total of at least five glycoproteins were shown to be virus specific with molecular weights of 118K, 98K, 88K, 62K, and 45K (Grose, 1980). A purified virion preparation showed six glycoproteins designated gp1 through gp6 with similar molecular weights (Shiraki et al.,1982).

Further studies have suggested that the envelope contains only three glycoproteins and that the others represent various stages of glycosylation (Okuno et al.,1983, Grose and Friedricks, 1982). However there is disagreement on just which these three are. Grose (1980) claims molecular weights of 118K, 98K, and 62K while Takahashi's group reports them as gp2 (83K,94K), gp3 (64K), and gp5 (55K,45K). Monoclonal antibodies developed against the 62K-64K glycoprotein have been shown to completely neutralize the virus suggesting a target for humoral immunity (Edson et al., 1984, in press). However others have suggested another polypeptide at 90K as the neutralizing antigen (Grose, 1980; Kamiya et al., 1982) and it is clear that even at this very basic level there exists very little agreement amongst contributors to VZV polypeptide analysis. The 62K-64K major glycoprotein along with the 92K glycoprotein was also shown to be sensitive to tunicamycin indicating the presence of N-asparagine-linked oligosaccharides. The 118K glycoprotein that Grose (1980) reports as a major envelope glycoprotein has also been suggested by Takahashi's group to be a precursor to the 62-64K glycoprotein (Okuno et al., 1983). Both groups seem to agree that there is a 45K glycoprotein that comigrates with actin and is shed from infected cells along with the 118-115K glycoprotein (Yamanishi et al.,

1981; Grose and Friedricks, 1982; Shiraki and Takahashi, 1982).

From the above review it is obvious that the characterization of the proteins of VZV is just beginning. To help increase the knowledge of the molecular biology of VZV it is often helpful to compare what is known about other herpesviruses, particularly HSV-1 and HSV-2. The following is an overview of HSV with references to more detailed reviews.

HSV Proteins

In contrast to VZV, countless papers have been published on the proteins and glycoproteins of HSV. Several excellent reviews of HSV are in print (e.g. Spear and Roizman,1980; Hay and Watson 1983). Table 3 summarizes the polypeptide distribution of HSV-1. As with some other viral systems, nomenclature has yet to be standardized and there currently exist five separate nomenclature systems for the identification of the structural polypeptides of HSV. VZV is not yet afflicted with this problem, except perhaps for the glycoproteins, since molecular weights are used for the most part.

The core and capsid of HSV possess six to seven polypeptides of which 60% is VP5 (155K), the major capsid protein (Gibson and Roizman, 1972; Cohen et al.,1980). Electron microscopy of negative-stained nucleocapsids has shown an organization similar to that described for VZV. The major capsid protein appears to be arranged in 162 capsomeres of which 150 are hexameric having dimensions of 9.5 x 12.5 nm with a channel 4nm in diameter running through a portion of the capsomere (Wildy et al., 1960). The remaining capsomeres, positioned at the vertices of the icosahedron, are presumed to be pentameric but the structure and dimensions are not yet known. Recent high resolution

electron microscopy combined with X-ray crystallography of polyoma virus, a smaller naked icosahedral capsid DNA virus, has challenged the contact theories of capsomeres in icosahedral symmetry. Polyoma virus was thought to have a typical hexameric and pentameric design but was instead shown to have an all pentameric capsid (Rayment et al.,1982; Harrison, 1983). None of the herpesvirus nucleocapsids have been subjected to high resolution electron microscopy to determine if the hexameric-pentameric model is still valid.

The functions of the other proteins that comprise the capsid are not clear, although a small 12K polypeptide may have a function in DNA condensation since it is phosphorylated and may be a DNA binding protein (Knopf and Kaerner, 1980). Others may be involved in the integrity of the capsid by formation of disulfide bonds (Zweig et al.,1979). Also a capsid protein designated P40 (VP22) has been shown to contain type- common as well as type-specific epitopes between HSV-1 and HSV-2 and may also be involved in capsid organization (Zweig et al.,1980). Other capsid proteins that seem to share antigenic determinants are the major capsid protein (VP5, 155K) and two other polypeptides of 50K and 40K (Hay and Watson,1983).

The major constituents of the envelope of HSV are the glycoproteins, of which there are five major species. These are commonly called gpA, gpB, gpC, gpD, and gpE. Their molecular weights are shown in Table 3. Although all their functions are not known, many studies on these glycoproteins have revealed some interesting features. Glycoprotein E has been shown to bind the Fc portion of Ig (Bauke and Spear, 1979) and is on the surface of infected cells as well as in the envelope. Glycoprotein D is the major glycoprotein associated

Polypeptides of HSV-1 virions

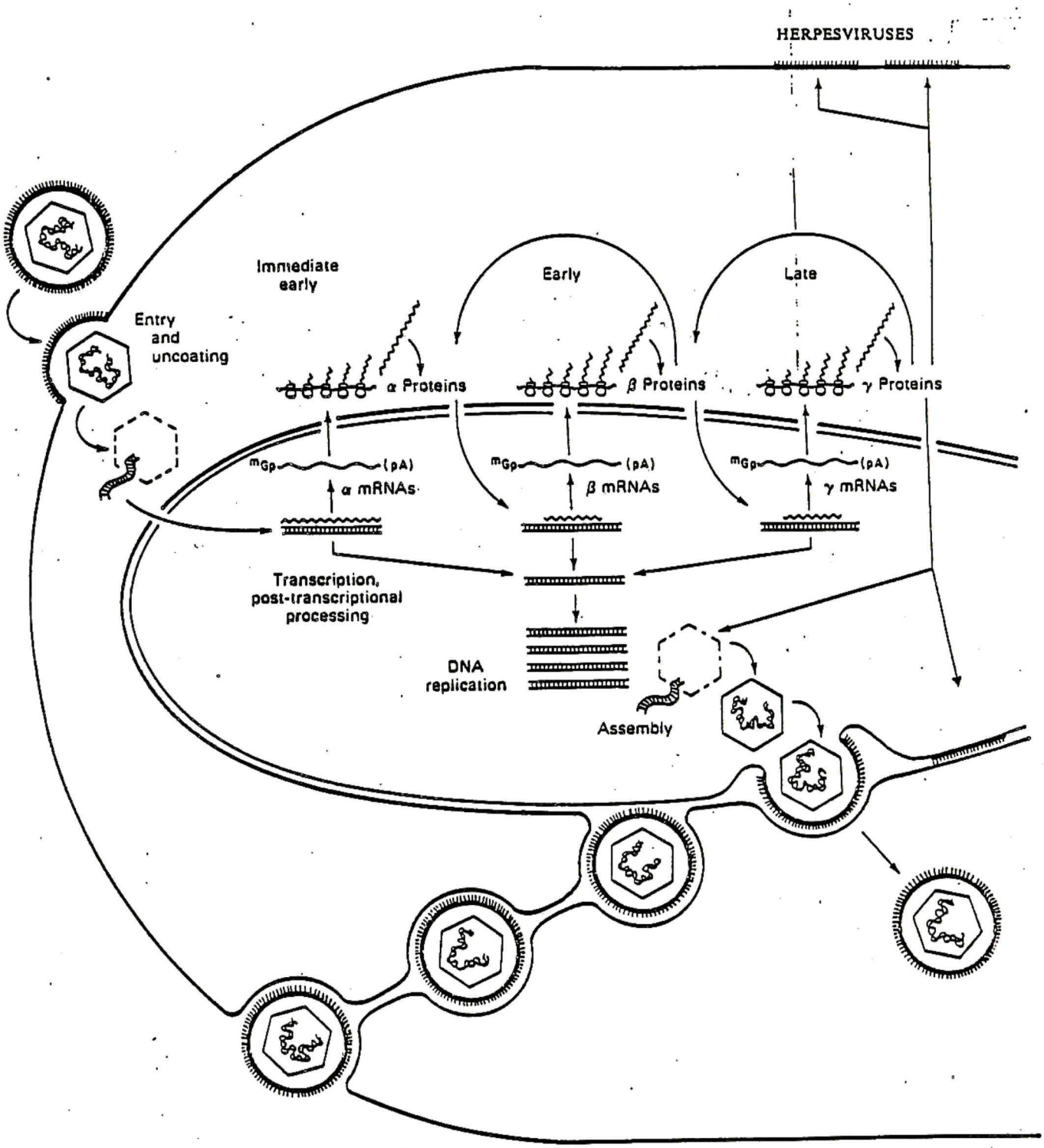
Polypeptide	Apparent molecular weight ($\times 10^{-3}$)	Location in virion
1–2	>260	
3	>260	
4	177 (162)	
5	157 (151)	nucleocapsid
6	149 (148)	
6.5	130	
8 (gC)[a]	129	envelope
7 (gB)	126	envelope
8.5 (gA)	119	envelope
9 (g)	115	
10	100	
11 (g)	94	
12	91	
12.3 (g) \ gE	88	envelope
12.6 (g) / gE	86	envelope
13	82	
14	80	
14.5 (g)	76	
15	73	
15.5 (g)	70	
15.8	69	
16	68	
17 (g) \ gD	62	envelope
18 (g) / gD	59	envelope
19E (g)	57	envelope
19C	55	nucleocapsid
20 (g)	51 (50.5)	
21	47 (42.5)	nucleocapsid
22	39 (37.5)	nucleocapsid[b]
23	36 (34.5)	nucleocapsid
24	<30	nucleocapsid

Table 3. Reproduced from Spear and Roizman (1980). Glycosylated polypeptides are indicated by the letter g.

with neutralization and has a similar molecular weight to the glycoprotein in VZV that also appears to be associated with neutralization (Grose, 1980; Grose et al.,1981). Penetration of absorbed virions appears to be mediated by gpB through its participation in cell fusion (Sarmiento et al., 1979). The functions of gpC and gp A are not yet clear, although gpC has been suggested to block cell fusion (Ruyechan et al., 1979) and gpA seems to be an alternate glycosylated form of gpB (Eberle and Courtney, 1980).

Dynamics of herpesvirus protein synthesis

Upon infection with HSV a cell undergoes numerous alterations in its protein synthesizing mechanisms. There is a rapid decline in host cell protein synthesis concomitant with the production and translation of virus mRNA. Subsequent virus protein synthesis is under a cascade type of control mechanism with at least three kinetic groups of polypeptides designated α , β , and γ (Honess and Roizman, 1974). The α group, whose mRNA synthesis does not require any de novo virus protein synthesis, reaches its peak of synthesis 3-4 hours post infection and subsequently induces the synthesis of the β group of polypeptides (Honess and Roizman, 1974). Most of the β polypeptides are phosphorylated (Knopf and Kaerner, 1980) and are DNA binding proteins (Hay and Hay, 1980). The β group inhibits most of the α synthesis while playing a role in DNA replication (Conley et al., 1981). This leads to the production of the γ group which contains mostly the structural polypeptides and are synthesized in increasing amounts until 12-15 hours post infection (Honess and Roizman, 1974). There is also a feedback inhibition of the transcription of β mRNAs by the γ group of polypeptides. The above sequence of events are diagrammed in Figure 2.



Sequence of events in the multiplication of herpes simplex virus from entry of virus into the cell by fusion of the virion envelope with the cell plasma membrane to assembly of virions and their exit from the cell through the endoplasmic reticulum. Also illustrated are transcription and coordinated sequential processing of mRNAs and synthesis of sets of proteins ($\alpha \rightarrow \beta \rightarrow \gamma$) required for DNA replication and virion structures. (Modified from diagram kindly supplied by B. Roizman, University of Chicago.)

Figure 2. Reproduced from Davis, Dulbecco, Eisen, and Ginsberg (1980).

Recent studies suggest that the above temporal scheme is more complex than originally suggested (Jones and Roizman, 1979;Zweig et al., 1980; Hay and Watson, 1983). Nevertheless it is a good model for understanding HSV polypeptide production. There is no current evidence to show whether VZV utilizes this kind of mechanism for control of polypeptide production.

In addition to the three classes of proteins discussed above another class of proteins, termed "heat shock" or stress proteins, has been shown to be activated in response to viral infections (Nevins, 1982, Notarianni and Preston, 1982). The heat shock response was first reported in 1962 on the puffing pattern of Drosophila chromosomes and is now regarded as a general homeostatic mechanism induced by a wide range of stimuli (Ashburner, 1982). In HSV-1 infected cells stress proteins of 90K, 70K, and 25K were detected and possibly activated by one or a number of "immediate early" (IE) polypeptides of the group (Notarianni and Preston, 1982). No such stress proteins have been reported for VZV.

Another important property of some HSV coded proteins is the ability to bind DNA. This binding has been shown in a number of herpesviruses to be necessary for regulatory functions during the expression and replication of the viral genome as well as the packaging of DNA into the nucleocapsid (Bayliss et al., 1975, Hay and Hay, 1980). HSV has approximately 12-16 DNA binding proteins which are present in all the temporal classes discussed above. A number of these undergo phosphorylation-dephosphorylation events which change their DNA affinity (Wilcox et al., 1980). The HSV DNA binding protein most extensively studied is a 128K species designated ICP8 that has antigenic sites in

common with similar proteins in cells infected with equine abortion virus, pseudorabies virus, or bovine mammillitis virus (Yeo et al., 1981; Blair and Honess, 1983). There are no reports in the literature on the DNA binding proteins of VZV.

HSV genome organization

The mapping of the gene organization of HSV has been done through the isolation of mutants, intertypic recombinants, and DNA transformation. These studies have been summarized in Figure 3 (Hay and Watson, 1983). For VZV such a picture is not at present possible since none of the techniques just mentioned have yet been successfully used, due to the cell associated nature of the virus.

Overall the HSV genome has a molecular weight of 100×10^6 daltons and a high G+C content of 67-69% (Becker et al., 1978). It has unique long (UL) and unique short (US) regions regions with terminal and internal inverted repeat sequences (TRl/IRl and TRs/IRs: see Figure 1). A consequence of this structure is that the DNA can undergo recombination at the L-S junction allowing UL and US to invert relative to each other and give rise to four equimolar populations of DNA (Wadsworth et al., 1976). VZV also demonstrates this isomerization (Straus et al.,1981) but as mentioned previously a left terminal repeat structure for VZV has not been clearly detected (Davidson, 1983). To what purpose these viruses perform this "flip-flopping" is not known.

A large number of HSV ts mutants have been produced and arranged into complementation groups, thereby allowing a number of genes to be identified (Schaffer et al.1978; Jofre et al.,1981). By analysis of the polypetides specified by intertypic recombinants of known gene parentage, some 30 or more polypeptides have been physically mapped

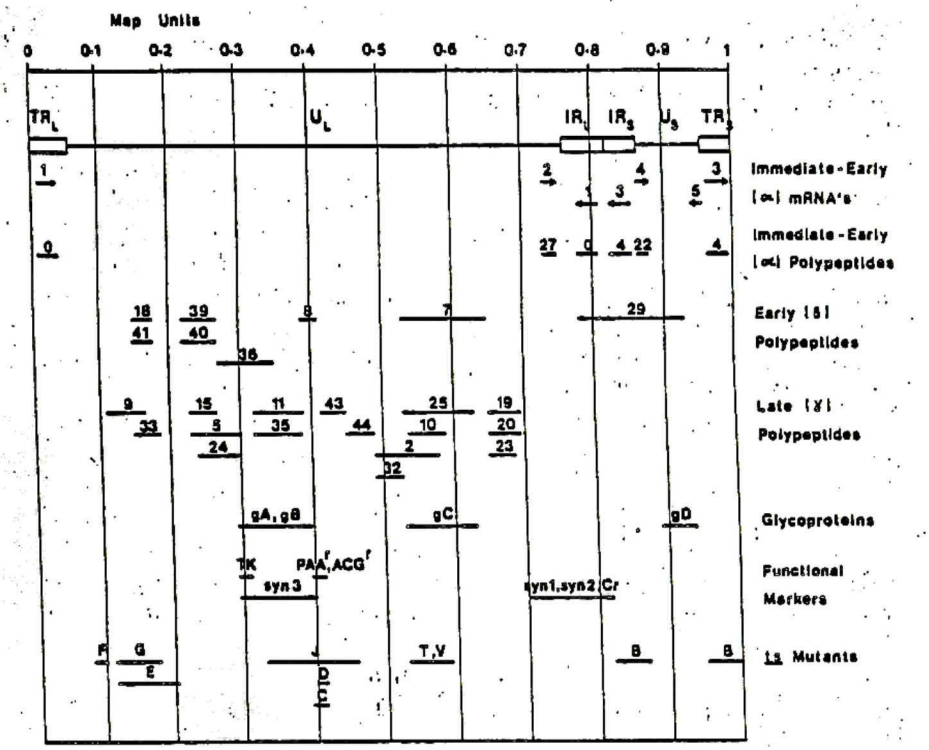


Figure 3 HSV-1 gene organization. The map locations and direction of synthesis of immediate-early transcripts (1 through 5) are based on data of Watson et al. [224] and Clements et al. [221]. The map locations of immediate-early polypeptide genes are based both on transcript mapping and in vitro translation data of Watson et al. [224] and Anderson et al. [223], while the location of early and late polypeptide genes are based on the intertypic recombinant analyses of Morse et al. [55]. The polypeptide nomenclature of the last authors has been used for all groups of polypeptides. Map location of the functional markers (syn 1, syn 2, syn 3, and Cr) are based on data of Ruyechan et al. [57], while those of genes conferring resistance to phosphonoacetate (paa^r) and acylcoguanosine (acg^r) are from Chartrand et al. [208] and Crumpacker et al [209], respectively. The thymidine kinase (TK) gene locus is derived from the marker transfer analyses of Wigler et al. [238] and the loci of HSV-1 strain KOS mutations are based on data of Parris et al. [206].

Figure 3. Reproduced from Hay and Watson (1983).

(Marsden et al., 1978; Morse et al., 1978; Halliburton, 1980). Viral transcripts, mainly the immediate early mRNAs, have been mapped and the direction of transcription studied (Clements et al., 1977; Mackem and Roizman, 1980; Watson et al., 1982). Direct gene transfer experiments have also been employed to map the thymidine kinase (TK) gene of HSV. Using either UV irradiated virus (Munyon et al., 1971; Allen et al., 1978) or transfection of cloned restriction endonuclease fragments (McDougall et al.,1980; Pellicer et al.,1978; Kit et al., 1980), mouse L cells lacking the enzyme deoxythymidine kinase (TK- phenotype) were converted to a TK+ phenotype via hypoxanthine, aminopterin, and thymidine (HAT) selective media. Although VZV has been shown to induce its own thymidine kinase (Kit et al., 1974; Cheng et al., 1979; Kallender et al.,1982) the location of this gene as well as all the other genes of VZV is unknown.

Aim of this dissertation

The main goal of this dissertation is to increase the knowledge of the molecular biology of VZV through the characterization of the polypeptides of this significant human pathogen. As discussed above very little has been published about this area of VZV pathogenesis. If meaningful research is to continue, the polypeptides of VZV need more study. To accomplish this a cell culture system will be investigated for its suitability for the growth of VZV and intracellular and virion associated virus-specific proteins will be analyzed. This will require the development of an improved virion isolation technique in order to distinguish virus-specific polypeptides from uninfected cell polypeptides. Various radiolabeling techniques will also be used to dis-

tinguish phosphoproteins and glycoproteins. Nonspecific cellular responses to viral infection will also be studied by identifying any stress or heat shock proteins in the culture system.

Once these proteins are catalogued, an investigation into their functions will be conducted using DNA cellulose chromatography to identify the DNA binding proteins of VZV. In conjunction with this, a new technique of protein-DNA binding on nitrocellulose paper will be used to augment the findings of the DNA cellulose chromatography. The drug cycloheximide will also be used to further identify "immediate early" polypeptides which may be important in establishing a VZV infection. These results should begin to identify proteins that are important in early molecular events during infection as well as those involved in DNA replication and packaging.

The immunological aspects of some VZV proteins will be explored using the Western technique of antigen-antibody interaction. Since zoster immune globulin (ZIG) is currently being used therapeutically, knowledge of antigenic sites would be helpful in understanding the immune response to VZV.

Finally experiments will be performed in an attempt to map the thymidine kinase gene to a specific portion of the VZV genome using DNA transfection and dot blot hybridization techniques.

II MATERIALS AND METHODS

Cells and Viruses

Cells used in the propagation of virus were human foreskin fibroblast cell line USUHS 184 (HFF) generously donated by Dr. G. Fisher, Dept. of Pediatrics, USUHS. Cells were grown in Eagles minimum essential medium supplemented with gentamycin (100 ug/ml) and 10% fetal calf serum (MEM-FCS). Mouse LTK- cells were generously donated by Dr. B. Moss, NIAID, NIH, Bethesda, MD and grown in MEM-FCS with occasional passage in MEM-FCS supplemented with 20 ug/ml of 5-bromodeoxyuridine (BUdR; Sigma Chemical Co., St.Louis, Mo.). Primary human embryonic lung fibroblasts (Flow 5000 cell line, Flow Laboratories, Rockville, MD), human esophageal cell line HEAA 29, and human esophageal SV 40 transformed cell line HESV, were provided by Dr. S. Straus, NIAID, NIH, Bethesda, MD.

VZV strains Ellen, Oka and Webster were obtained from the American Type Culture Collection, Rockville, MD. VZV strain Scott was a clinical isolate provided by Dr. G. Fisher, Dept. of Pediatrics, USUHS. VZV strain Champ was a clinical isolate provided by Dr. A. Buchan, The University of Birmingham, U.K.

Passage of VZV was accomplished by the addition of freshly harvested infected cells to confluent monolayers of HFFs at a multiplicity of one infected cell per six uninfected cells ("MOI" of 1:6). Infected cultures were grown at 37°C in either 150-cm^2 flasks or 850-cm^2 roller bottles until the appropriate cytopathic effects (CPE) were present. The medium was decanted, and infected cells were scraped, suspended, and dispersed by gentle mixing in a small volume of fresh

medium.

For herpes simplex viruses, HSV-1 strain 17 (Brown et al.,1973) and HSV-2 strain HG-52 (Timbury, 1971) were used. Unless otherwise noted these HSV strains were grown on confluent monolayers of HFFs using a multiplicity of infection (MOI) of 0.1-0.05 PFU per cell. Infected cells were harvested in the same manner as described above when the appropriate CPE was present.

Confluent monolayers of uninfected cells were treated in the same manner as infected cells with the obvious exception of virus. Such "mock infections" were incorporated into experiments for control purposes and labeled either "M" for mock infected or "U" for uninfected controls. Unless specifically noted, equal amounts of uninfected and infected cells were used in all experiments.

Adenovirus type 2, vesicular stomatitis virus (VSV) Indiana strain, and coronavirus MHV A59 that were used in the agarose gel electrophoresis experiments were the generous gifts of Dr. John McGowan, Dept. of Microbiology, USUHS (McGowan et al., 1982).

Nucleocapsid purification

VZV and HSV nucleocapsids were isolated by a method described by Straus et al. (1981). Infected cell monolayers were washed twice with phosphate buffered saline (PBS) at 4°C, then scraped down and pelleted at 2000 rpm for five minutes in an IEC PR6000 centrifuge. The cell pellet was cycled three times through freezing in a slurry of dry ice and acetone and thawing at 37°C. The pellet was then suspended in 0.5 ml of lysis buffer for each 75 to 150 cm^2 of cell monolayer harvested. The lysis buffer contained 0.5% Nonidet P-40, 3.6 mM calcium chloride, 5 mM magnesium acetate, 125 mM potassium chloride, 0.5 mM EDTA [pH7.5],

6 mM B-mercaptoethanol, and 0.5% deoxycholate. To each 1 ml of lysis buffer-cell pellet solution was added 25 ug (approximately 50 U) of DNase I and 25 ug of RNase A (both from Worthington Diagnostics, Freehold, N.J.). The solution was incubated at 37°C for 30 min and extracted once with an equal volume of genosolv-D (trichloro-trifluoro-ethane; Allied Chemicals, Morristown, N.J.). The mixture was shaken for one minute and centrifuged at 1000 rpm for 10 min at 4°C. The upper aqueous phase was removed and layered over a discontinuous gradient of 5% and 40% glycerol in lysis buffer made up in SW-40 nitrocellulose centrifuge tubes (Beckman Instruments Inc, Palo Alto, Calif.). The gradient was centrifuged at 40,000 rpm for 45 min at 4°C using an SW-40 rotor in a Sorvall OTD ultracentrifuge. The supernatant was discarded, and the pellet containing the viral nucleocapsids was resuspended in an appropriate solution. For DNA purification 2% sodium dodecyl sulfate, 0.1 M Tris-hydrochloride [pH7.5], 20 mM EDTA,(2 x STE buffer), was used. For electron microscopy pellets were resuspended in 10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA (TE buffer).

Virion purification

VZ virions were purified by a unique combination of the methods described by Dumas et al.(1980) and by Shemer et al.(1980) with several modifications. When VZV infected cells reached the appropriate CPE, usually 90-95%, they were washed twice with PBS, 4°C, scraped and pelleted at 4°C by centrifugation at 2000 rpm for 5 min in an IEC PR 6000 centrifuge. The cell pellet was resuspended in 1 ml of cold PBS for each 75-150 cm^2 of cell monolayer harvested. This cell suspension was then homogenized with a Tissuemizer (Tekmar Co, Cincinnati, Ohio) for 30 sec at 4°C. The homogenate was then centrifuged at 1500 rpm for

10 min at 4°C and the supernatant was prepared for the first stage of purification involving zone centrifugation. The supernatant was ultrasonically disrupted with a twenty second burst from a Heat Systems Ultrasonics sonicator (output setting 5) and carefully layered onto a continuous 5% to 55% sucrose gradient in TBS buffer (0.05 M Tris-hydrochloride, 0.15 M NaCl, pH 7.4). PBS may be used as an alternate buffer. The gradients were centrifuged at 20,000 rpm for 1 hr at 4° C using an SW-27 rotor in a Sorvall OTD ultracentrifuge. A broad band was visualized starting approximately 3-4 cm from the bottom when the tube was illuminated from below. The band was collected with a 10 cc syringe fitted with a 19-gauge needle inserted into the side of the tube. The band was diluted with cold TBS and pelleted at 20,000 rpm at 4°C, for 1 hour using the SW-27 rotor and ultracentrifuge mentioned above. The pellet was resuspended in 4 ml of TBS and sonicated as above.

The second stage in purification involved the use of positive density, negative viscosity glycerol-tartrate gradients (Obijeski et al.,1974). With this method gradients are formed with increasing density toward the bottom of the tube, due to increasing tartrate concentration, and decreasing viscosity, due to decreasing glycerol concentration. The resuspended virus from the sucrose gradients mentioned above was layered onto continuous 30% glycerol - 50% potassium tartrate (w/w) gradients in TBS. The gradients were then centrifuged for 17 hours at 20,000 rpm using an SW-27 rotor in a Sorvall OTD ultracentrifuge. Two bands were visible approximately 4-5 cm from the bottom of the tube. The lower band contained only enveloped VZV whereas the upper band contained a mixture of membranes, nucleocapsids, and partially enveloped virions as determined by examination in

the electron microscope. The lower band was collected by side puncture, diluted in TBS and rebanded on another glycerol-tartrate gradient as before. This resulted in one band of highly purified VZ virions (see Figure 20 in Results section). This band was removed by side puncture, diluted in TBS, and pelleted as before at 4°C, in an SW-27 rotor, 20,000 rpm for 1 hour. The supernatant was discarded and the pellet prepared immediately for polyacrylamide gel electrophoresis, electron microscopy, or frozen at -70°C.

Polyacrylamide gel electrophoresis

Separation and detection of proteins by polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulphate (SDS) and N,N' diallytartardiamide (DATD) cross-linked slab gels as described by Laemmli (1970) and Heine et al.(1974). SDS-PAGE was performed on vertical slab electrophoresis units (Hoefer Scientific) with water cooling. Gels were prepared at room temperature by mixing an appropriate volume of stock acrylamide (28% w/v) and DATD (0.74%) solution with other stock solutions to give a final mixture containing 12% acrylamide, 0.4 M Tris-HCl (pH8.9), 0.03% N N N'N' tetramethylene diamine (TEMED), 0.1% SDS and 0.36% ammonium persulfate. The gels were then poured between glass plates with appropriate spacer strips, usually 0.7 or 1.5 mm thick, and allowed to polymerize with a water overlay. Gel were either 10 cm, 18 cm, or 25 cm in length. After polymerization the water overlay was removed and a stacking gel mixture was poured between the plates and a comb inserted to allow sample well formation. The stacking gel consisted of 4.5 % acrylamide, 0.1% DATD, 0.12 M Tris-HCl [pH 7.0], 0.03% TEMED, 0.07% ammonium persulfate and 0.1% SDS.

Unless specifically noted, equal amounts of cells were used in preparing samples. Samples were prepared for SDS-PAGE by solubilization in a "disruption buffer" consisting of 0.05 M Tris-HCl [pH 7.0], 4% sucrose, 2% SDS, 5% B-mercaptoethanol, and 50 ug/ml Bromphenol blue. They were sonicated for 30-60 sec and heated for 45-60 sec at 100 ^{o} C. Samples were then applied to the wells and overlayed with electrophoresis buffer consisting of 0.025M Tris-glycine pH 8.5, and 0.1% SDS. This buffer was used in both the upper and lower reservoirs. Electrophoresis was performed at 50 volts per gel until the dye front had passed through the stacking gel and entered the running gel. The voltage was then increased to 200 - 250 volts and run for 3-4 hours. Heat was dissipated by constant water cooling at 14 ^{o}C. If no water cooling was available, gels were run at 50 volts each for 12-18 hours. When the dye front was within 2 cm of the bottom of the plates the electrophoresis was halted. Molecular weight standards (New England Nuclear Corp, Boston, MA) were routinely used on all gels.

Fixing, Staining, and Autoradiography

Gels were removed and fixed for silver staining according to the method of Merril et al.(1981) with modifications. Gels were fixed in a solution of 50% methanol-12% acetic acid overnight with two changes. The gels were then rinsed with three changes of 10% ethanol-5 % acetic acid, 10 min each with constant rocking throughout the rest of the procedure. Gels were soaked in a fresh solution of 0.0034 M potassium dichromate and 0.0032 N nitric acid for 5 minutes. They were washed four times, for 30 sec each, in 200 ml of deionized water and placed in 200 ml of 0.012 M silver nitrate for 30 min. The gels were washed once for 30 sec in 200 ml of deionized water followed by one rapid rinse

with 200 ml of developer solution containing 0.28 M sodium carbonate and 0.5 ml of formalin per liter. The gels were agitated in a second portion of this solution until the image had reached the desired intensity. Development was stopped by discarding the developing solution and adding 100 ml of 1% acetic acid. This procedure was limited to gels less than 1 mm thick.

For gels of greater thickness the silver stain procedure of Wray et al. (1981) was used. Gels were soaked overnight in 50 % methanol with three changes. The stain was prepared by mixing solution "A" (0.8 g silver nitrate in 4 ml distilled water) dropwise into solution "B" (21 ml of 0.36% sodium hydroxide and 1.4 ml of 14.8 M ammonium hydroxide) and then increasing the volume to 100 ml with distilled water. The gels were stained in this solution for 15 min with constant agitation. The gels were then washed in distilled water for 5 min while a fresh developer solution was prepared. The developer solution consisted of 2.5 ml of 1% citric acid and 0.25 ml of formalin in 500 ml of water. The gels were gently agitated in this developer for 10-15 min until the bands had reached the desired intensity. Staining was stopped by placing the gel in 50% methanol.

Gels were dried under vacuum and heat on a slab gel drier (Hoefer Scientific) using Whatman No. 17 filter paper as a backing. Autoradiography was carried out using Kodak XAR-5 film and cassettes.

Electron microscopy

Tissue culture specimens in 100 mm petri dishes were prepared for electron microscopy by decanting the media and adding 1% glutaraldehyde in PBS at 4°C. After four min the plates were scraped with a rubber policeman and placed in a conical centrifuge tube. The cells were spun

at 1500 rpm for 10 min at 4°C in an IEC 6000 centrifuge. The supernatant was carefully decanted and 2% glutaraldehyde in PBS, 4° C, was added to the pellet. After 15 min the pellet was dislodged and transferred to a Wheaton snap-cap glass vial filled with PBS. After five min the PBS was changed and further operations carried out under a chemical hood. The PBS was replaced by 1% osmium tetroxide in PBS for 30 min. The pellet was then dehydrated according to the following schedule: 50% ethanol, two changes 15 min each; 70% ethanol, 15 min; 95% ethanol, 15 min; 100% ethanol, 2 changes, 15 min each; propylene oxide, 30 min. The pellet was soaked overnight with two changes of a 1:1 mixture of propylene oxide: Epon 812. The next day the pellet was placed into a pool of Epon 812 and trimmed into 1 mm^3 blocks with an acetone cleaned razor blade. The blocks were transferred to Epon filled beem capsules and oriented at the bottom with a wooden stick. The specimens were cured overnight at 65°C. After curing the specimens were removed from the beam capsules, trimmed, and sectioned on a Sorvall MT 5000 Ultramicrotome. Sections were picked up with 400 mesh EM copper grids and stained with 2% aqueous uranyl acetate for 5 min, washed, stained in 0.3% lead citrate, washed and dried by blotting with Whatman # 3 filter paper.

Specimens were negatively stained by applying a drop of viral preparation for 60 sec to a 400 mesh EM cooper grid bearing a nitrocellulose substrate (Formvar) covered with a thin layer of carbon. The grid was washed twice with drops of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0), and stained for 15 sec with 1% aqueous uranyl acetate. The stain was wicked off with Whatman #3 filter paper and allowed to dry.

Specimens for cryo-electron microscopy were prepared by absorption of viral nucleocapsid preparations for 60 sec to a 400 mesh EM copper grid bearing a nitrocellulose substrate (Formvar) covered with a thin layer of carbon. Following the method of Kistler et al. (1977), the grid was washed four times with drops of glass distilled water and immediately immersed in liquid nitrogen. While under liquid nitrogen the specimens were loaded into a Balzers High Vacuum Freeze Etch Unit BAF 301. After ice sublimation specimens were shadowed with platinum and slowly returned to room temperature.

Specimens were viewed and photographed with a Philips EM 400T electron microscope operating at 80 kV. Micrographs were recorded on Kodak 4463 film and developed for 4 min in Kodak D19 developer diluted 1:1 with water at 20°C, using an Arkay EM410-4 Film Processor. Some EM facilities and technical assistance were generously supplied by Dr. Alasdair Steven, Laboratory of Physical Biology, NIH, Bethesda, MD.

Radioactive labeling of polypeptides

Most proteins were labeled with ^{35}S-methionine (New England Nuclear Corp, Boston, MA) at 8-20 uCi/ml. The radioactive media was the usual MEM-FCS with 1/4th the normal cold methionine concentration and an appropriate amount of ^{35}S-methionine added. At the beginning of a labeling period, the normal media was removed and the radioactive media added. At the end of the labeling period the radioactive media was removed and the cultures were washed twice with cold PBS prior to scraping and pelleting.

Glycoproteins were labeled with a mixture of ^{3}H-glucosamine and ^{3}H-mannose (New England Nuclear) at 5-10 uCi/ml each in MEM-FCS. A ^{14}C-glucosamine and ^{14}C-mannose mixture at 1-2 uCi/ml was also used to

label glycoproteins. After the labeling periods the cultures were treated similarly to the ^{35}S-methionine labeled cultures.

Phosphoproteins were labeled with ^{32}P orthophosphate (carrier free, New England Nuclear Corp) at 20-40 uCi/ml in phosphate free MEM-FCS. Prior to labeling cultures were incubated in the phosphate free media for one hour. Cultures were then treated as mentioned above.

Sulfated proteins were labeled with ^{35}S-sodium sulfate (I.C.N., Irvine, Calif.) at 20 uCi/ml in MEM-FCS. Cultures were treated similarly to those for ^{35}S-methionine labeling.

Pre/Post labeling experiment

Two groups of HFFs (#184) were set aside and marked "pre-label" and "post-label". The prelabel cells were immediately placed into ^{35}S-methionine media, 8-10 uCi/ml, for 72 hours while the post-label cells were allowed to continue growth in regular media. The media in the prelabel cells was then changed back to normal "cold" MEM-FCS for 24 hours with two changes. Cells in both groups were infected with VZV (strain Oka) at an MOI of 1:6. When the cells showed 50% CPE (36 hours post infection) the media in the post-label group was replaced with ^{35}S-methionine media. At 90-95% CPE the cells in both groups were harvested and the virions isolated as previously described using sucrose and potassium tartrate-glycerol gradients. The proteins were then analyzed on 12% SDS-polyacrylamide slab gels as described previously.

Agarose gel electrophoresis

VZV, HSV-2, Adenovirus, VSV and Coronavirus were subjected to agarose gel electrophoresis in a manner similar to that done with bacteriophage T-7 capsids (Serwer and Pichler, 1978). The nucleocapsids

of VZV and HSV-2 (strain HG 52) were prepared as mentioned above. Adenovirus type 2, VSV Indiana strain, and Coronavirus MHV-59 were prepared and donated by Dr. J. McGowan, USUHS. The samples were suspended in small volumes of Tris/Mg buffer (0.2M NaCl, 10 mM Tris-HCl, 1 mM MgCl , pH 7.4) and diluted prior to electrophoresis in 5 volumes of tracking buffer (2% sucrose, 5 mM sodium phosphate [pH 7.4], 1mM MgCl , 400 ug/ml bromophenol blue). A 0.9% low melt agarose (F.M.C. Corp, Rockland, ME.) horizontal gel in 0.05 M sodium phosphate, 1 mM MgCl , pH 7.4, was constructed in a mini-gel electrophoresis model H6 system (Bethesda Research Laboratories, MD) and the samples were carefully loaded into the wells. Electrophoresis was performed at 50 V (constant voltage) with circulation of the electrophoresis buffer (0.05 M sodium phosphate, 1 mM MgCl , pH 7.4) for 90 minutes at room temperature. Gels were then stained in 0.05% Coomassie Blue, 10% acetic acid for 2-3 hours. The gels were destained by diffusion into 10% acetic acid overnight. For autoradiography gels were dried under vacuum and heat on Whatman No.17 filter paper and exposed to Kodak XAR-5 film as mentioned previously.

Nucleocapsid trypsin treatments

The nucleocapsids of VZV and HSV-2 were isolated as described above (Straus et al., 1981) and resuspended in TE buffer (10 mM Tris-Hcl, 1mM EDTA, pH 7.4). 50 ul of nucleocapsid suspension was mixed with 50 ul of a PBS solution containing 9 units of trypsin activity (Worthington Diagnostics). Reactions were stopped at various times by the addition of 100 ul of 2X disruption buffer and immediately placing the sample in a boiling water bath for 1 minute. Samples were subjected to SDS-PAGE analysis as described above.

For electron microscopy samples were treated with trypsin as above except that the reaction was stopped by the addition of 50 ul of aprotinin (Sigma Chem. Co.). The samples were then spun for 2 minutes in a Beckman Microfuge B and the pellet was resuspended in TE buffer. Samples were prepared for electron microscopy by the negative staining technique described above.

DNA cellulose chromatography

Radiolabeled extracts of HFF monolayers infected with VZV strain OKA were used throughout the DNA cellulose chromatography experiments. HFF monolayers were infected with the usual "MOI of 1:6" and when 25-30% CPE was apparent, the media were replaced with media containing ^{35}S-methionine (8-20 uCi/ml). Incubation was continued until the cells showed 90-95% CPE. The infected cell monolayers were washed twice with PBS at 4° C, then scraped and pelleted at 2000 rpm for five minutes in an IEC PR6000 centrifuge. For specific labeling of phosphoproteins the cells were infected as above. At 25-30% CPE the cells were washed twice with phosphate free media for one hour and then incubated in the presence of medium containing 20-40 uCi/ml of ^{32}P orthophosphate. The cells were harvested as described above. Cell pellets were stored at -60° C prior to processing for DNA cellulose chromatography.

Harvested cell pellets were thawed and all subsequent operations were performed at 4° C. Cells were resuspended in sonication buffer (0.05 M NaCl, 0.01M MgCl , 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.05 M Tris-HCl pH7.6, 130 ug/ml lysozyme, 23 ug/ml phenylmethylsulphonyl fluoride, 20 ug/ml DNAse I, 0.4% sodium deoxycholate) at a concentration of 3 x 10^7 cells/ml (final volume approximately 5 ml). The cells were then ultrasonically disrupted with

three twenty second bursts from a Heat Systems Ultrasonics Sonicator (output setting 5). The extract was incubated at 4°C for two hours. Following incubation, NaCl was added to a final concentration of 2.0 M. Incubation was continued for an additional sixty minutes. DNA and precipitated proteins were removed by centrifugation at 16,000 rpm using a Sorvall RC5 centrifuge. The supernatant was dialyzed overnight against three changes of Buffer A (0.05 M NaCl, 1 mM EDTA, 1mM 2-mercaptoethanol, 0.05 M Tris-HCl pH 7.6).

Native and denatured calf thymus DNA cellulose were prepared by Dr. W. Ruyechan, Dept. of Biochemistry, USUHS, using the method of Alberts and Herrick (1971). 2 cm x 0.5 cm columns were used throughout these studies and the columns with the DNA cellulose were equilibrated in Buffer A at 4°C prior to use. Half of the dialyzed extracts was then applied to each column. The columns were washed extensively with Buffer A and eluted by the stepwise addition of 0.2, 0.6, 1.0, and 2.0 M NaCl in Buffer A. A 4.0 M guanidine hydrochloride wash was also performed on some columns in an attempt to strip off any residual, tightly bound proteins.

Column eluates were collected in 2 ml fractions and the radioactivity of a small aliquot (usually 50 ul) was measured in a Beckman LS 9000 Liquid Scintillation counter. Fractions of peak radioactivity from each of the salt steps were concentrated by overnight precipitation at 0 C following the addition of one-tenth volume of an "S-100" unlabeled protein extract (Hay, 1979) from uninfected cells and four volumes of acetone. The "S-100" protein extract was prepared by washing HFF monolayers twice with cold PBS scraping, pelleting as before and allowing the cells to swell in 1 ml of distilled water per 100 mm petri

dish of cells for fifteen minutes at 4°C. After vortexing, an equal volume of a buffer containing 4 M NaCl, 0.04 M Tris-HCl pH 8.2, 2 mM EDTA, and 2 mM 2-mercaptoethanol was added to lyse nuclei. The samples were incubated on ice for thirty minutes, then centrifuged at 100,000 x g for one hour at 4°C. The supernatant was removed and dialyzed overnight against 0.05 M NaCl, 0.02 M Tris-HCl pH 8.2, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol. A light precipitate was removed from the dialysate by centrifugation at 10,000 x g for thirty minutes.

The acetone precipitated proteins from the column eluates were collected by centrifugation at 3000 x g for fifteen minutes. The precipitate was solubilized in disruption buffer, heated at 100° C for 45-60 sec and subjected to SDS-PAGE as previously described.

Blotting of VZV polypeptides to DNA

Infected cells, nucleocapsids, virions, and other samples in disruption buffer were electrophoresed on 1.5 mm 12% SDS polyacrylamide slab gels using a vertical, water cooled SE 620 slab gel electrophoresis unit (Hoefer Scientific). Following electrophoresis the gels were transferred to a TE 52 Transphor apparatus (Hoefer Scientific) for electrophoretic transfer of proteins onto nitrocellulose filters (BA 85, Schleicher & Schuell). The transfer buffer used was 25 mM Tris-HCl, 0.192 M glycine, 20% methanol, and electrophoresis was carried out at 1.5 amps, 60 volts for 2-3 hours with water cooling. Following transfer, the nitrocellulose sheet was soaked in 200 ml of binding buffer (1 mM EDTA, 10 mM Tris-HCL pH 7.0, 50 mM NaCl, 0.02% bovine serum albumin, 0.02% polyvinylpyrollidine, 0.02% ficoll) (Bowen et al., 1980) for at least 30 min. The nitrocellulose was transferred to a Seal-A-Meal cooking pouch. Ten ml of binding buffer containing one of a

variety of ^{32}P labeled DNAs (approximately 10^5 cpm/ml) were added. Air bubbles were removed and the pouch was incubated for a minimum of two hours at room temperature with constant agitation on a rocking platform. Binding was terminated by removal of the radioactive binding mixture followed by four 15 min. washes with cold binding buffer. The washes were monitored for radioactivity to insure complete removal of non-bound probe. The nitrocellulose sheet was then air dried and prepared for autoradiography as described above.

If necessary the bound DNA could be removed from the nitrocellulose sheet by soaking in several changes of 2 M NaCl in binding buffer overnight with constant agitation on a rocking platform. The amount of DNA still bound was monitored by autoradiography. The nitrocellulose sheet could then be reprobed with a different DNA sample in the same manner as described above.

Occasionally the efficiency of protein transfer was tested by running duplicate blots and staining the nitrocellulose filter immediately after electrophoretic transfer with Napthol Blue Black (0.1%, 45% methanol, 10% acetic acid) for one hour. Destaining was carried out by diffusion into 90% methanol, 10% acetic acid.

Cycloheximide treatment of infected cells

Monolayers of HFFs in 100 mm petri dishes were infected with VZV strain OKA at an "MOI" of 1:3. After one hour the inoculum was removed and MEM-FCS containing 50 ug/ml of cycloheximide was used to wash the cells and then added to the cells for a five hour incubation. Cells were released from the cycloheximide block by removing the medium, washing the monolayer 4 times with normal MEM-FCS, and incubating with normal MEM-FCS. At appropriate times after washing, the medium was

replaced with ^{35}S-methionine medium containing 20-30 uCi/ml for 150 min. time periods. The radioactive medium was then removed and the cells harvested as described previously for SDS-PAGE analysis. Variations in the above procedure included using sonicated infected cells as the inoculum and blocking the infected cells with cycloheximide for 7, 10, 15, and 20 hours. As controls, a normal time course of VZV polypeptide synthesis was run simultaneously.

Heat shock proteins

Uninfected HFF monolayers were grown in 175 cm^2 plastic tissue culture flasks (Costar #3150). They were heat shocked by floating in a circulating water bath at 43°C for one hour. Fresh medium at 37°C was then substituted and the cells were returned to 37°C. At 1-3, 3-6, and 6-8 hours post heat shock the medium was substituted with ^{35}S-methionine media at 20-30 uCi/ml. Cells were then harvested in the routine manner for SDS-PAGE analysis.

Western blot analysis

Non-radioactive samples were prepared and subjected to 12% SDS-PAGE on 1.5 mm thick vertical slab gels as mentioned previously. Following electrophoresis, gels were transferred to a TE 52 Transphor apparatus (Hoefer Scientific) and the proteins were transferred to nitrocellulose filters in the same manner as described for the protein blots above. Electrophoresis was carried out at 1.0 amps, 30 volts, for 17 hours with water cooling. Following transfer the nitrocellulose filter was soaked in TNB buffer (3% BSA, 0.9% NaCl, 10 mM Tris-HCl pH 7.4) for 1 hour at room temperature. The filter was rinsed twice with distilled water and incubated, with agitation, in 10 ml of NETG-Aby buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.25% gelatin, 0.5% NP 40, 0.5 ml

Zoster immune globulin [ZIG]) for 2 hours at room temperature. The ZIG was generously donated by Dr. D. Waters, State Biologic Laboratories, Dept. of Public Health, Boston, MA. The blot was then washed ten times with 20-30 mls of NETG wash buffer (NETG, 0.5% Triton X-100, 0.1% SDS) and allowed to soak with gentle agitation for 2 hours with ^{125}I-Protein A in 10 mls of NETG, 0.05% NP 40 (10^6 dpm/10ml)(Towbin et al., 1979). The ^{125}I-Protein A was kindly provided by Dr. K. Holmes, Dept. Pathology, USUHS. After incubation the blot was rinsed ten times in NETG wash buffer, air dried and set up for autoradiography. To test efficiency of protein transfer, a duplicate gel was run and stained with Napthol Blue Black (0.1% Napthol Blue Black, 45% methanol, 10% acetic acid) for one hour and destained by diffusion into 90% methanol, 10% acetic acid.

In addition to ZIG, a rabbit hyperimmune serum to HSV-1 infected human embryonic lung cells was used in a Western blot analysis performed with Dr. A Buchan, Dept. Medical Microbiology, Medical School, Birmingham, U.K. Three different VZV isolates and ^{35}S-methionine labeled VZV proteins were provided to test cross reactions between HSV and VZV.

Immunoprecipitation

Antigen for immunoprecipitation studies was prepared by resuspending radiolabeled cells (infected and uninfected) in a small volume (usually 2 ml) of RIPA buffer (0.5 M NaCl, 0.1% SDS, 1 mM PMSF, 1% sodium deoxycholate, 1mM methionine, 10 mM Tris-HCl pH 7.4)(Okuno et al., 1983) and sonicated for 3 minutes in a Heat Systems Ultrasonics bath. All subsequent operations were performed at 4°C. Samples were incubated on ice for one hour and centrifuged at 30,000 rpm using a TI

60 rotor in a Sorvall OTD Ultracentrifuge. 100 ul of the supernatant was mixed with 200 ul of washed Cowan's Strain I Staphylococcus aureus (kindly provided by Dr. K. Holmes, USUHS) and 500 ul of 1% NP 40 in PBS for 10 min in order to preclear the antigen. Samples were then centrifuged at 3,000 rpm for 15 minutes resulting in approximately 600 ul of precleared antigen as the supernatant. The supernatant was reacted with 60 ul of a monoclonal antibody to the 115K glycoprotein of VZV generously provided by Dr. M. Zweig, NCI, Frederick, MD., for one hour. 200 ul of washed Stapylococcus aureus (Staph A) was added and incubation was continued for 10 minutes. The samples were centrifuged at 3000 rpm for 10 minutes and the pellet was washed and repelleted with 1 ml of 0.05% NP 40 in PBS. The pellet was solubilized in disruption buffer, heated in a water bath at 37° C for 15 min., and centrifuged at 3000 rpm, 4°C, for 10 min. The supernatant was stored at -20°C or immediately subjected to SDS-PAGE electrophoresis.

Preparation and labeling of DNAs

Most of the DNAs used in the protein blots, dot blots, and transfections were prepared and generously donated by Mr. Thomas Casey, a fellow graduate student at USUHS. The VA gene of adenovirus cloned in PBR 322 was kindly provided by Dr. John McGowan, Dept. of Microbiology, USUHS. VZV DNA was purified from nucleocapsids as described by Straus et al. (1981) and phage DNA was purified as described by Vande Woude et al. (1979). Briefly nucleocapsids or phage were suspended in 2X STEP buffer (2% SDS, 0.1 M Tris-HCl pH 7.5, 20 mM EDTA, 1 mg/ml proteinase K) for 30 min at 50 C. The DNA was extracted twice with redistilled phenol saturated with 0.2 M Tris-HCl (pH 7.9) followed by an extraction with a 50:50 phenol-chloroform mixture. Two final

extractions were done with equal volumes of chloroform. The upper aqueous phase was carefully removed and precipitated in 3 volumes of absolute ethanol at -20°C. The DNA was pelleted by centrifugation at 10,000 rpm for 30 min at 4°C.

In vitro labeling of DNA was accomplished by the method of Rigby et al. (1977) using a BRL nick translation kit #8160 (Bethesda Research Laboratories, Bethesda, MD). Z-DNA [poly(dG-dC)] was purchased from P-L Biochemicals (Milwaukee, Wis.) and labeled in vitro by phosphate transfer from [^{32}P]ATP with T4 polynucleotide kinase. The specific activities of radiolabeled DNAs were measured with a Beckman LS 9000 liquid scintillation counter.

Dot blot hybridization

To test VZV DNA for homologous gene sequences of HSV-1 and HSV-2 thymidine kinase (TK), an SRC-96 Minifold dot hybridization apparatus (Schleicher & Schuell, Keen, N.H.) was used. The DNA to be probed was obtained from a library of cloned EcoRI and BamHI VZV digests available in this laboratory (Straus et al., 1982). HSV-1 and HSV-2 TK DNA was kindly donated by Dr. G. Hayward, Dept of Pharmacology, Johns Hopkins University School of Medicine, Baltimore,MD. and used as probes.

Blot hybridizations were performed according to instructions supplied by Schleicher & Schuell. 5 ug of the DNA to be probed was diluted to 170 ul with 100 mM Tris-HCl and placed into a 1 ml Eppendorf tube. 30 ul of 2N NaOH and 100 ul of 20X SSC (3 M NaCl, 0.15 M sodium citrate) were added, and the mixture was then heated at 80 C for 10 min. The mixture was neutralized with 120 ul of 2 M Tris-HCl (pH 8.5) and absorbed onto nitrocellulose paper (BA 85, Schleicher & Schuell) using the SRC-96 Minifold apparatus. The nitrocellulose blot was air dried

overnight and then baked for 2 hours at 80°C. The blot was transferred to a Seal-A-Meal pouch and 10 ug of denatured salmon sperm DNA in 10 ml of hybridization buffer (6X SSC, 30% Formamide, 0.5% SDS, 0.02% ficoll, 0.02% polyvinylpyrollidine, 0.02% BSA) was added. The blot was then incubated for 72 hours at 56 C in a circulating water bath. 5-10 ug of ^{32}P-labeled probe was added and hybridization continued for another 72 hours. The blot was removed from the pouch and washed with 2X SSC, six times, 15 min each. It was allowed to air dry and prepared for autoradiography.

HSV-1 and HSV-2 TK genes were used as probes either in their plasmid carriers or after being cut out with restriction endonucleases. HSV-1 TK containing plasmid was cut with BamHI and HSV-2 TK containing plasmid was doubly digested with Hind III and Sal I. They were then electrophoresed on a 1% low melt agarose gel and the bands were extracted according to the CTAB (hexadecyltrimethylammonium bromide) method (Langridge et al., 1980) by Mr. Mark Wellman who also kindly nick translated the resulting DNA probes for blotting onto the nitrocellulose filters.

Transfection of DNA for thymidine kinase activity

The method used for transfection of DNA into mouse LTK- cells was the calcium phosphate precipitation technique (Graham and Van der Eb, 1973, McDougall et al.,1980). Mouse LTK- cells were passed by trypsinization into 60 mm petri dishes so that by the next day a 30-50% monolayer was present. To sterile 5 cc test tubes the particular DNA in TE buffer was added with carrier DNA (either salmon sperm or LTK- cell DNA) so that there would be a total of 20 ug DNA/ml. Hepes buffered saline (HBS,pH 7.05) was added to bring the volume to 950 ul. 50 ul of

2.5 M CaCl was added and the mixture was gently vortexed. After standing at room temperature for 20 min the mixture would form a faintly milky microprecipitate. The medium was removed from the cells and 0.5 ml of the mixture was added to each petri dish. The cells were incubated at 37°C for 45 min with frequent rocking. 5 ml of MEM-FCS was added and the incubation continued for 4 hours. A DMSO boost was then performed by decanting the media, washing the cells with HBS, and adding 2 ml of 15% DMSO in HBS to each plate for 1-2 min. The DMSO was quickly removed, and the cells washed twice with HBS. Fresh MEM-FCS was added and the incubation continued. The medium was changed again at 24 hours post boost and at 48 hours HATG selective media (10^{-4} M hypoxanthine, 10^{-6} M aminopterin, 4×10^{-4} M thymidine, 10^{-5} M glycine in MEM-FCS) was added to the cells. Cells were checked daily for the presence of colonies, and the HATG medium was changed every third day. DNAs transfected included the entire library of cloned VZV restriction endonuclease fragments, whole VZV DNA, HSV-1 and HSV-2 TK DNA. Negative controls consisted of carrier DNA only.

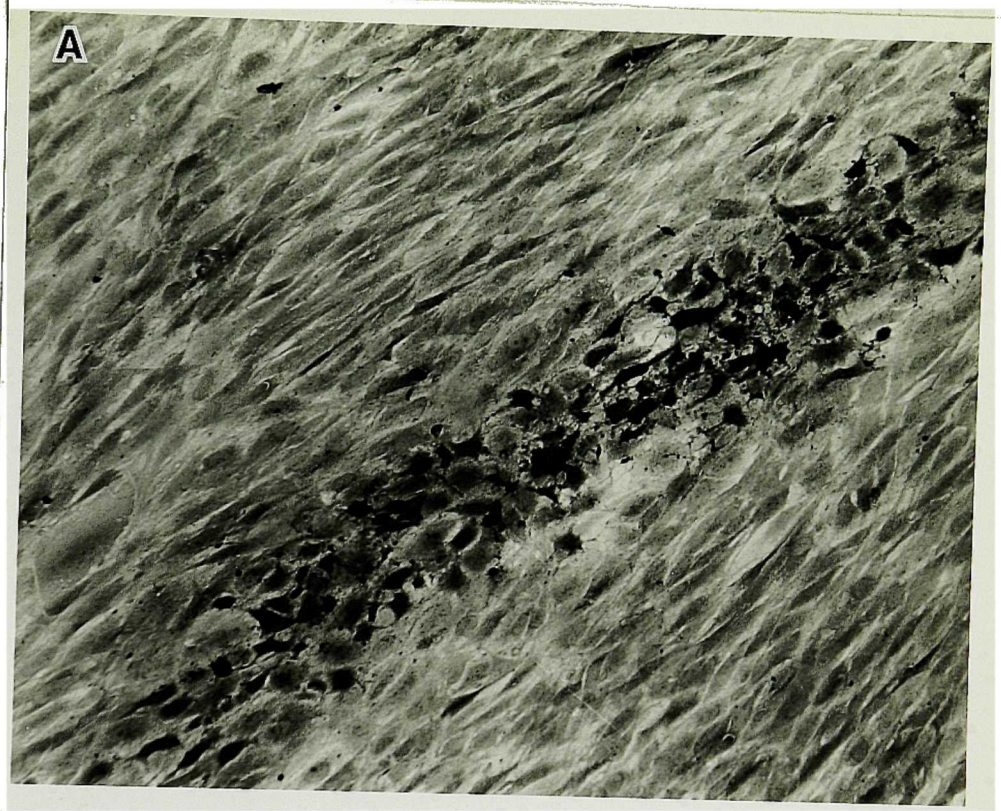
III RESULTS

Electron microscopy of VZV infected tissue culture cells

Since Weller in 1953 first propagated VZV in human embryonic skin-muscle tissue culture, other cell lines of human, monkey, and guinea pig have been found to support viral growth. Unfortunately the virus remains cell associated and slow growing. As a consequence the molecular biology of VZV has not been as thoroughly investigated as that of other herpes viruses. In an attempt to overcome some of the problems of tissue culture that have retarded the study of VZV, several new cell lines were investigated. These included human foreskin fibroblasts (HFFs), human embryonic lung fibroblasts, and two human esophageal cell lines. These cell lines were tested because they were, for the most part, easy to grow in tissue culture and formed monolayers suitable for infection. Since it is generally accepted that VZV spreads via the respiratory route, it was hoped that the human embryonic lung and/or the human esophageal cell lines would be more suitable for viral growth as they were derived from tissues that occupy the initial pathway of VZV infection.

We found that HFFs were the optimal cell culture system for the study of VZV. Typical plaques of VZV infected HFFs are shown in Figure 4. In the upper photograph (A) an early plaque (24 hours post infection) of VZV infected cells (darker stained) is surrounded by normal HFFs. In the lower photograph (B), a late plaque (5 days post infection) is shown with clear areas where VZV infected cells have died and detached from the monolayer. Using an "MOI" of one infected cell to

Figure 4. Photomicrographs showing typical cytopathic effects (CPE) of infection with VZV. In panel A, normal human foreskin fibroblasts (HFF), 24 hours post infection, surround an early plaque of VZV infected cells which appear rounded and more intensily stained. In panel B, a late plaque of VZV infected cells (5 days post infection) is shown with a clear central area where dead cells have detached from the monolayer. Cells were stained with crystal violet.
Magnification: X 200.


A

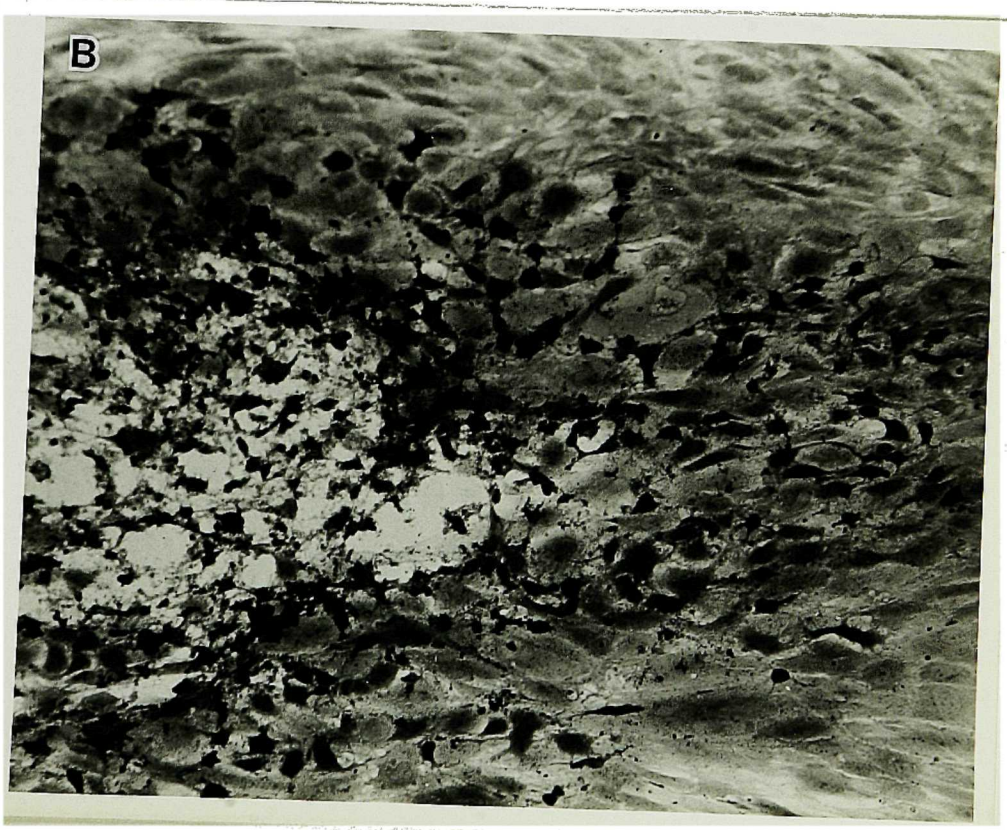
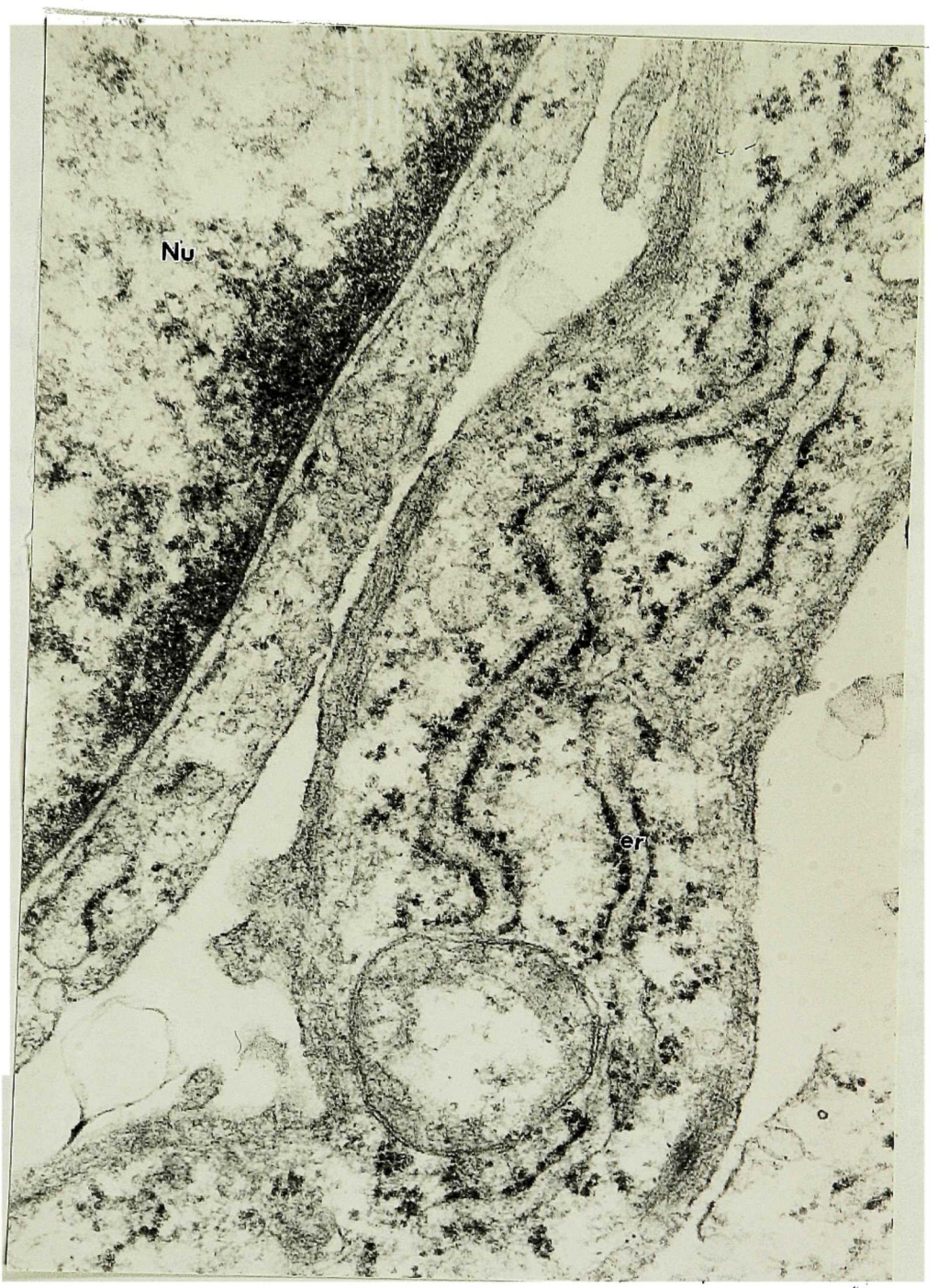

B

Figure 5. Survey electron micrograph of a thin section of an uninfected human foreskin fibroblast (USUHS cell line #184) with a portion of its cytoplasm folded upon itself. Nu, nucleus; er, rough endoplasmic reticulum. Magnification: X 43,000.


Nu
er

six uninfected cells, a monolayer of HFFs reach 90-95% CPE in 3-5 days.

Electron microscopy of HFFs revealed some interesting views of VZV infection. Uninfected cells are long and spindle shaped with a large nucleus that is centrally located and occupies approximately 1/4th of the cell volume. The cytoplasm shows typical fibroblastic morphology with rough endoplasmic reticulum, microfilaments, few mitochondria, and a poorly developed golgi apparatus. A typical uninfected HFF is shown in Figure 5. Cells were then viewed 23 hours after infection with VZV. In Figure 6 several viruses can be seen within the cell while nucleocapsids are in the nucleus. No heavily infected cells were observed passing virus to uninfected cells through cytoplasmic contact at this time.

At 96 hours post infection, when CPE was at 90-95%, the nuclei of infected cells contained a large number of nucleocapsids and the cells were breaking apart as a result of infection. Figure 7 shows a portion of the disrupted cell nucleus containing a cluster of nucleocapsids with varying core densities. The cytoplasm contained many enveloped virions but the enveloping process was not observed.

Human embryonic lung fibroblasts (Flow 5000 cell line) late after infection with VZV resembled the cell in Figure 8. The cell has almost completely broken apart and nucleocapsids are crowded in the nucleus with enveloped virions seen in the cytoplasm. However in this cell line, CPE at the light microscope level was difficult to distinguish from the uninfected state until late in infection (5 to 7 days post infection). In both HFF and Flow 5000 cell lines it appeared that a substantial amount of virus was released into the medium when the cell died. However attempts to spread the infection using the cell free

Figure 6. Varicella-zoster virus infected HFF cell 23 hours after inoculation. Several viruses (arrows) can be seen at the junction between cells. Magnification: X 20,000. Enlarged areas show nucleocapsids within the nucleus and viruses at the plasma membrane. Nu, nucleus. Magnification: X 110,000.

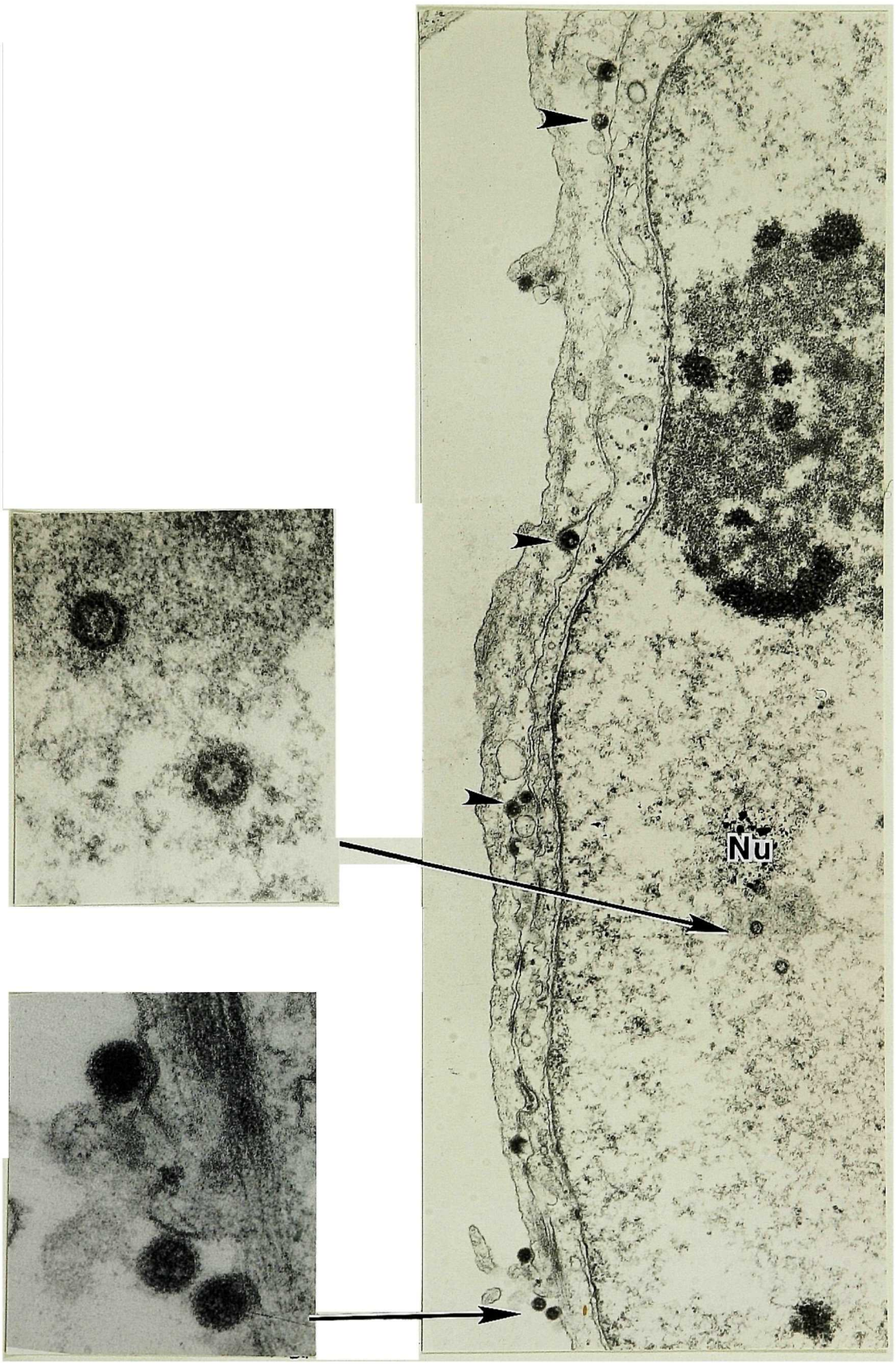

Nu

Figure 7. The disrupted nucleus of an HFF cell 96 hours post after inoculation with VZV. A large cluster of nucleocapsids with cores of varying densities is shown.
Magnification: X 60,000.

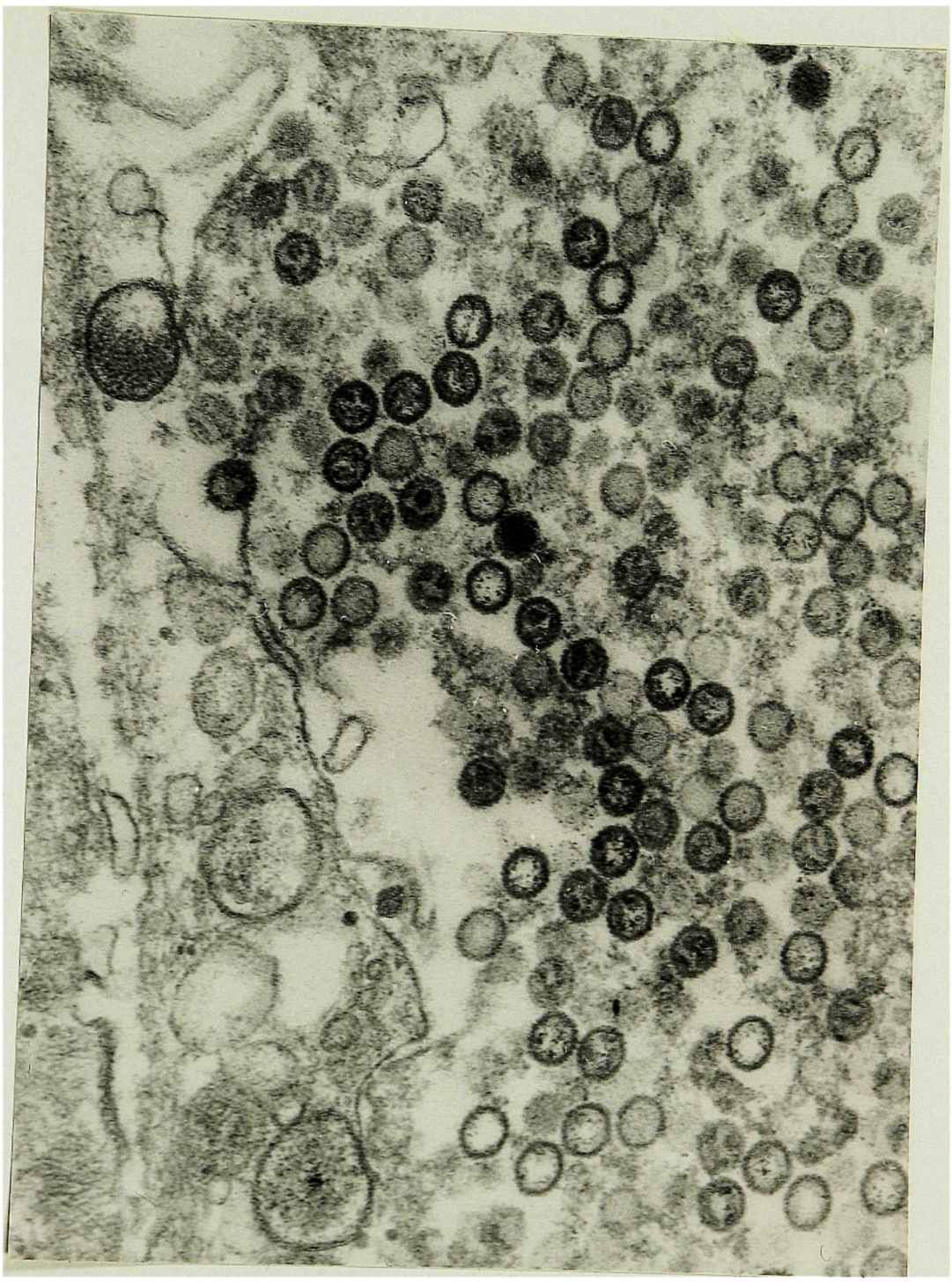
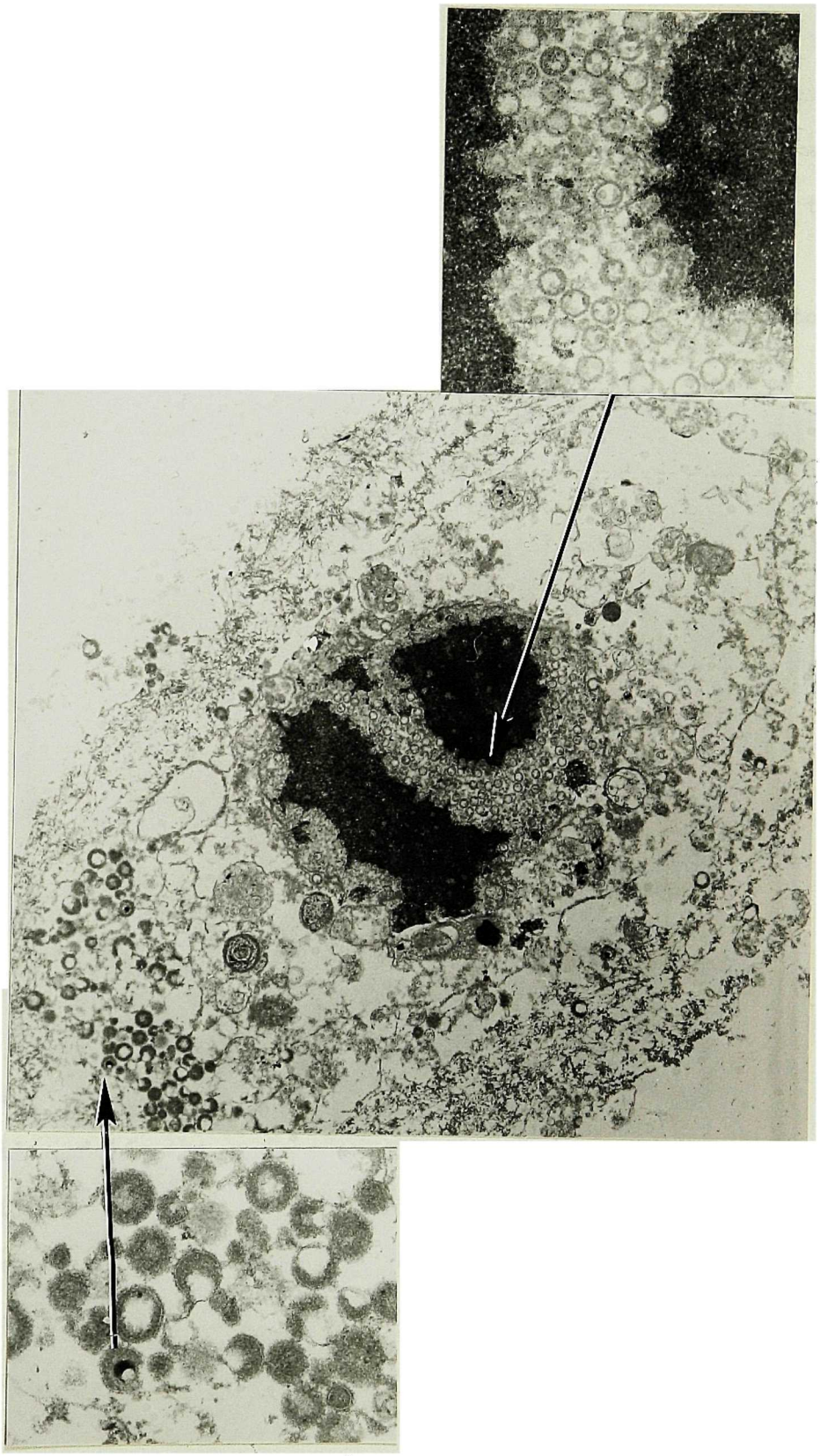


Figure 8. A disintegrating human embryonic lung fibroblast 96 hours after VZV inoculation. Magnification: X 16,000. Enlargements show nucleocapsids crowded in the nucleus (top, magnification: X 50,000) and enveloped virions in the cytoplasm (bottom, magnification: X 70,000).

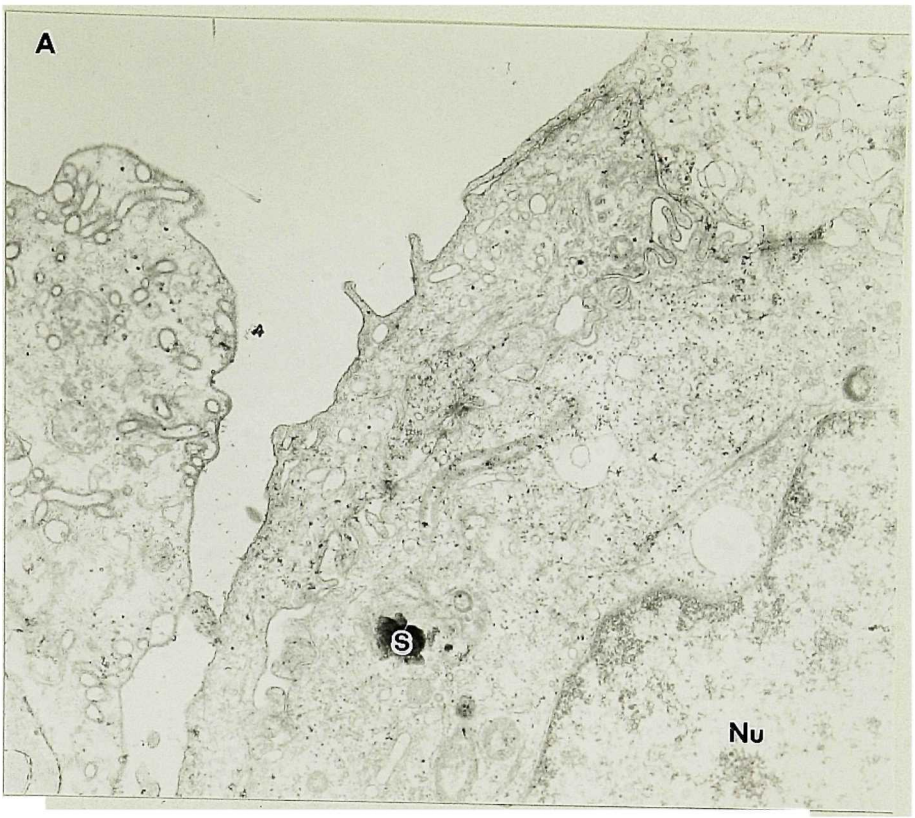


supernatant or sonicated infected cells proved very inefficient, but was possible (titers of 10^3 to 10^4 pfu/ml were obtained). Virions could be purified by centrifugation from medium over infected cell cultures, and this was used as a source of DNA and nucleocapsids although the preparation had a very low infectivity titer.

Since it is generally assumed that VZV spreads via the respiratory route, two human esophageal cell lines were also investigated as to their feasibility for growth of VZV. One was a human esophageal cell line designated HEAA 29 and the other was an SV 40 transformed human esophageal cell line designated HESV. These cell lines were infected with VZV by Dr. S. Straus, NIAID, NIH, Bethesda, MD., and after 17 passages they were used to inoculate uninfected cell cultures. Both infected and uninfected cell cultures were then examined by electron microscopy Uninfected HEAA 29 cells were mostly oval in shape with a large centrally located nucleus. Figure 9A shows a typical uninfected HEAA 29 cell. Uninfected HESV cells were more spindle shaped and contained a large oval nucleus (Figure 10). At 96 hours after inoculation with VZV infected cells comparatively little virus was seen in either cell line (Figures 9B and 11). Of the two cell lines, the HESV cells showed more virus. Figure 11 shows an HESV cell with approximately 35 nucleocapsids within the nucleus and several enveloped virions within the cytoplasm. However most of the HESV cells and the HEAA 29 cells showed no structural evidence of VZV infection at this time. Figure 9B shows one of the few HEAA 29 cells containing viral structures. Often no viral particles were seen in either cell line. Like the Flow 5000 cell line, the CPE in HEAA and HESV cells at the light microscope level was difficult to distinguish from the appearance of uninfected cell

Figure 9A. Survey micrograph of uninfected human esophageal cells (cell line HEAA 29). Nu, nucleus; s, stain precipitate. Magnification: X 18,000.

Figure 9B. A human esophageal cell (HEAA 29) 96 hours after VZV inoculation demonstrating the typical lack of virus production in this cell system. Magnification: X 18,000. Enlargement on the right (magnification: X 50,000) shows two nucleocapsids in the nucleus (Nu). Enlargement on the left (magnification: X 50,000) shows a virion in the cytoplasm and a virion at the plasma membrane (V). S, stain precipitate.


A
S
Nu

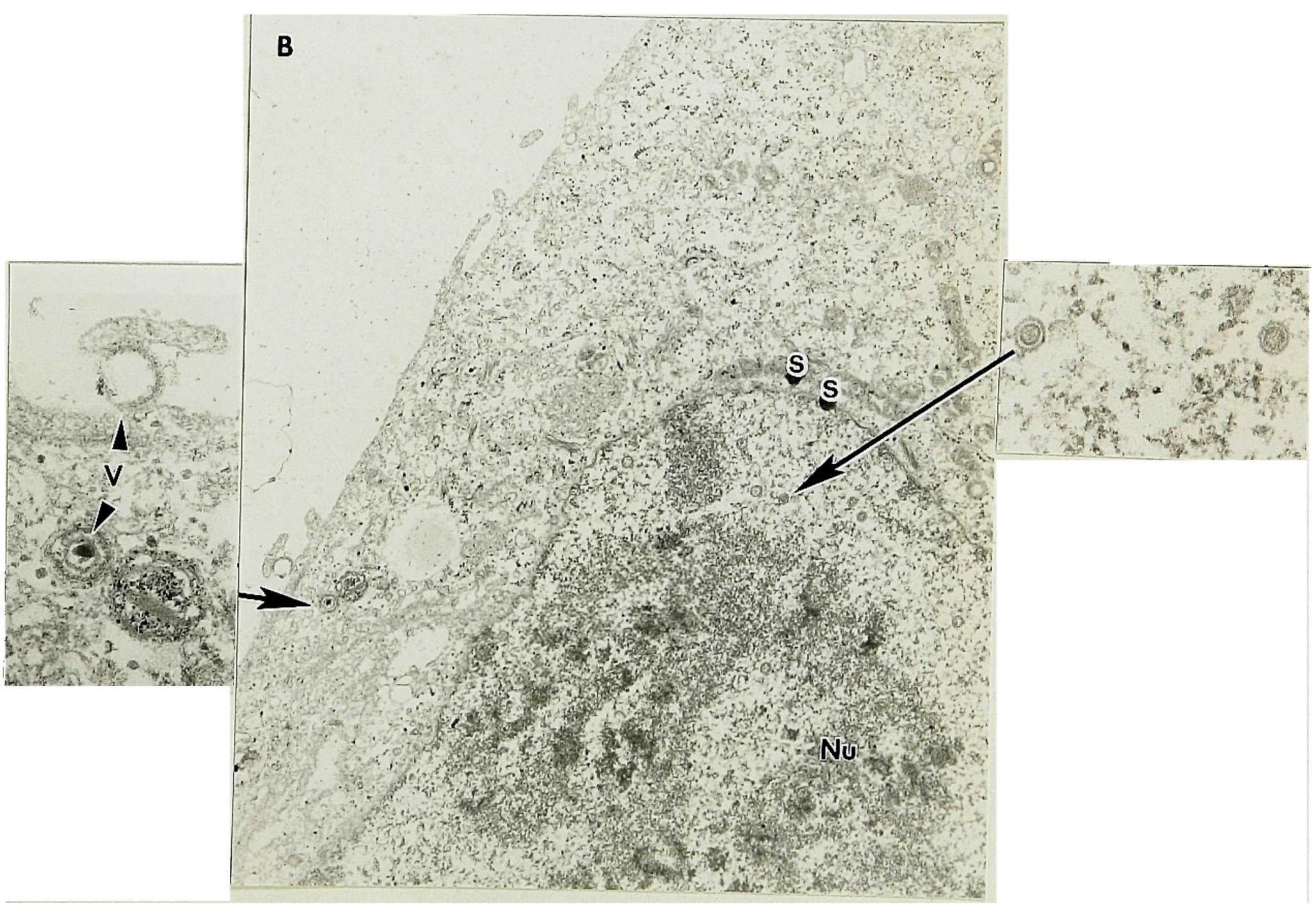

B
V
S
S
Nu

Figure 10 Survey micrograph of an uninfected HESV cell showing a large oval nucleus. Nu, nucleus. Magnification: X 18,000.

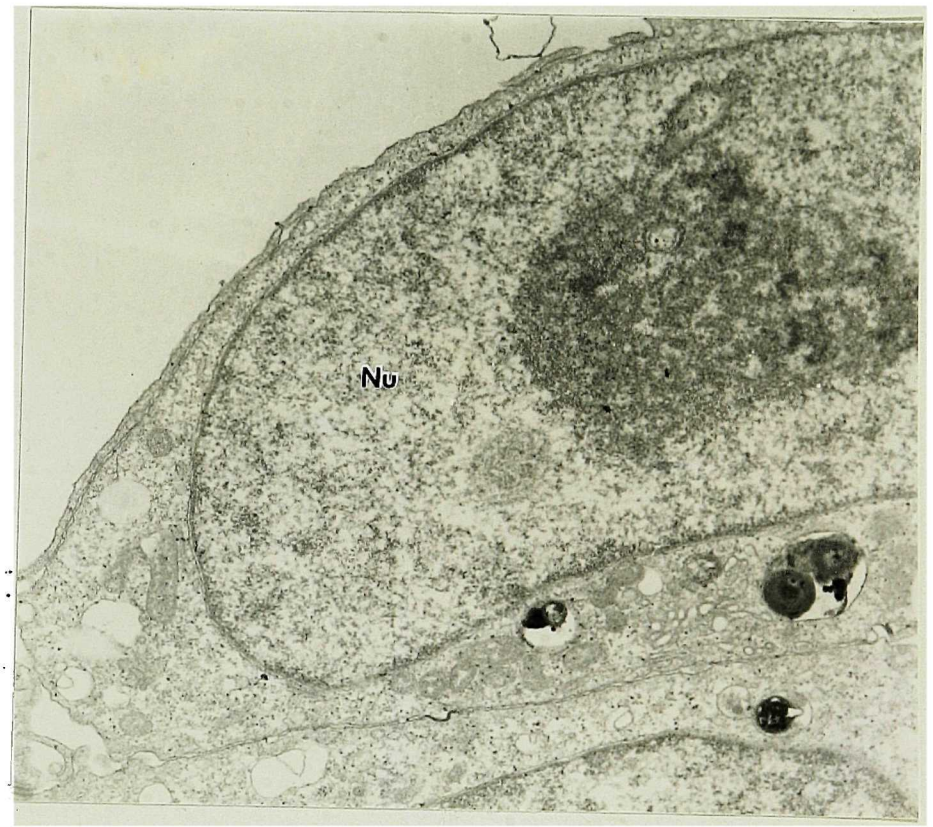
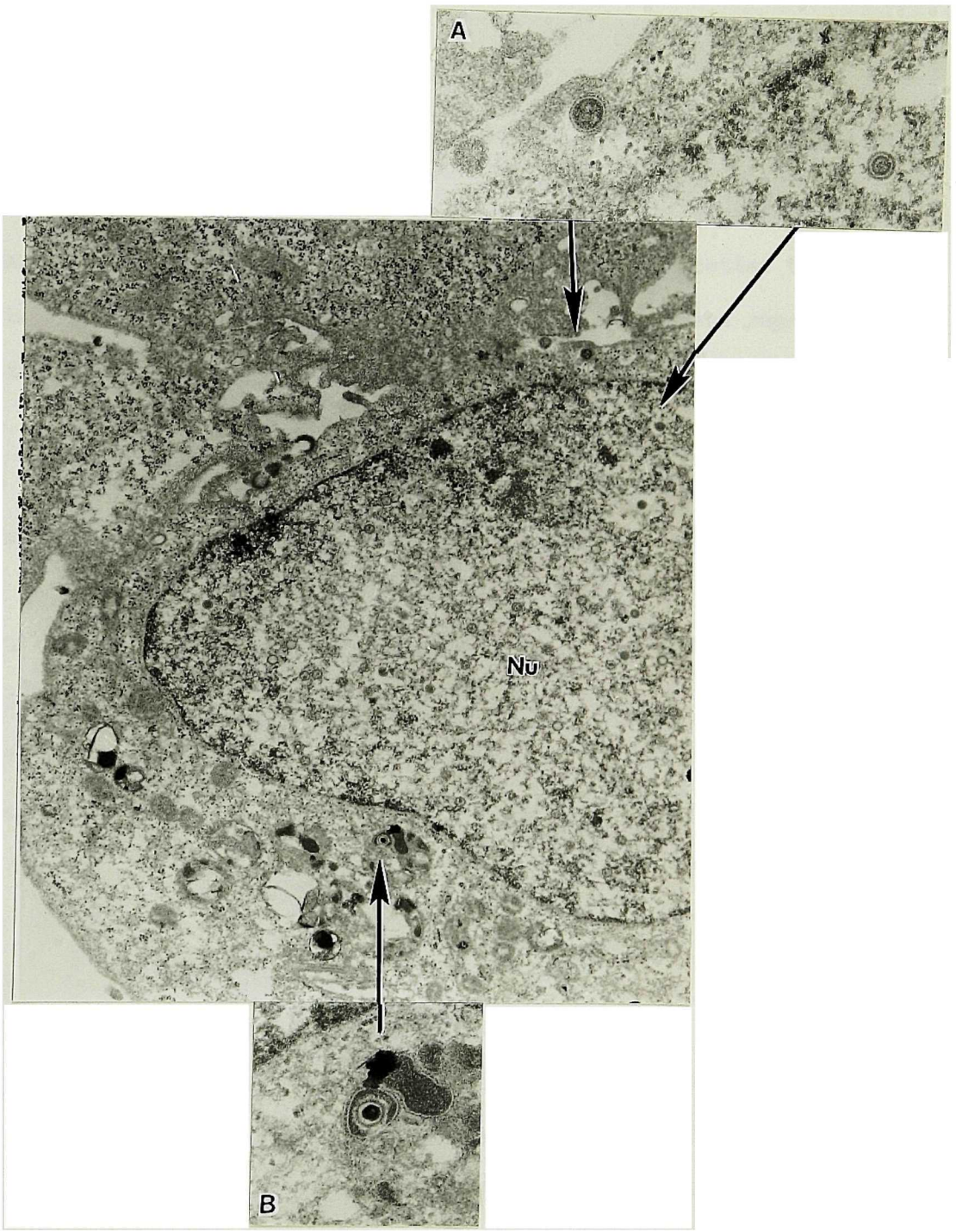

Nu

Figure 11. An HESV cell 96 hours after inoculation with VZV. Magnification: X 18,000. Enlargements (magnification: X 50,000) show virus within the cytoplasm and the nucleus (Nu). There are approximately 35 nucleocapsid structures within the nucleus in this thin section. An example of a nucleocapsid is shown in enlargement A (NC). A mature enveloped virion can also be seen (V). A virus particle is seen in enlargement B as it passes through the cytoplasm.


A
Nu
B

cultures although appearance of CPE may not necessarily correlate with viral yield. Of the cell lines tested, therefore, the HFF (184) line showed evidence for the largest amount of viral replication based on particles visible in infected cells.

Infectivity assays were carried out on sonicates of HFF cells infected with VZV which showed viral yields of 10^3 to 10^4 pfu/ml (per 10^7 cells). This level of viral production, although poor by other herpesvirus standards, is nevertheless as good as or better than most of the other cell types used to grow VZV which have been reported in the literature (Gershon et al., 1973; Mar et al., 1978; Grose et al., 1979; Neff et al., 1981; Sasaki et al., 1981; Gilden et al., 1982; Iltis et al., 1982; Takahashi, 1983).

Based on the above observations, therefore, the HFF cell line (USUHS #184) was judged to be optimal for the study of the molecular biology of VZV. HFF cells grow well to high passage, they replicate virus quite rapidly, and they give a yield of infectious VZV equivalent to the best reported in the literature (Grose et al., 1979; Takahashi, 1983).

One other aspect of the cell-virus system which was investigated was the optimal method of infecting cells for experimental purposes. Whole infected cells and several types of cell free extracts were used to infect HFFs. CPE and viral protein synthesis were then followed. In all cases where cell free preparations were used, the infectious process as judged by CPE and viral polypeptide appearance was slow (six days as opposed to 24 hours for whole cells) and asynchronous. Plaque assays showed that the best number of infectious centers we could achieve with cell free virus is no better than 1 pfu/ 10^3 cells using the supernatant from pelleted and sonicated infected HFFs. For this

reason, experiments were routinely carried out using whole infected cells as an inoculum.

Identification of VZV specific cell-associated and virion polypeptides

As a first step in understanding the molecular biology of VZV it was important to identify and catalogue the proteins involved with this pathogen. Although there are a few reports of VZV specific polypeptides in the literature (see Introduction), there is little general agreement on the number and sizes of viral proteins, and no information on their functions, with the exception of the glycoproteins. Thus it was necessary, using the HFF cell system and several strains of virus, to initiate a study of VZV by cataloging the proteins of infected cells and then identifying their structural and functional roles.

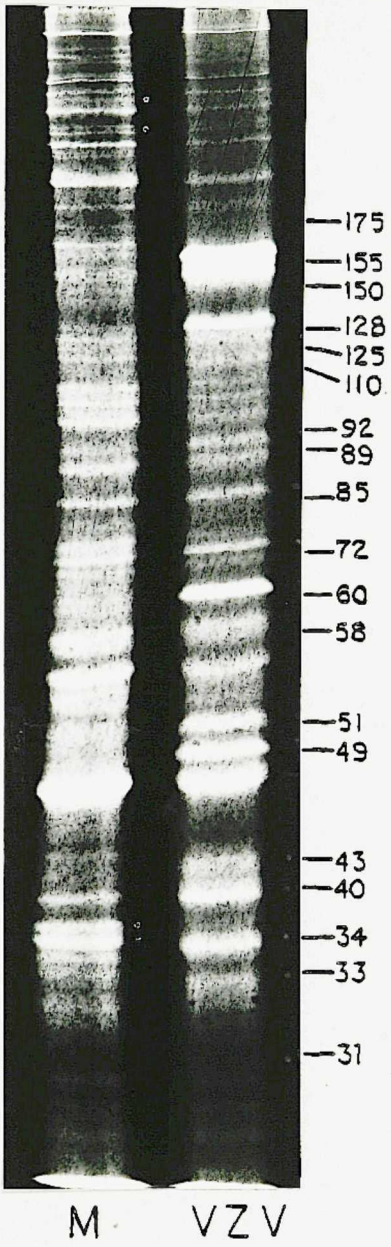
The general approach to be used in our studies was to first define a list of polypeptides uniquely associated with VZV infected cells. This set of polypeptides would be compared with those described in the literature prior to utilizing a number of techniques for characterizing them further. Those polypeptides associated with the virus structure would be identified using purified nucleocapsids and virions while glycoproteins would be visualized by means of specific carbohydrate labeling. Another important category of polypeptides which can be identified using a specific label is the phosphoproteins which would be detected using the incorporation of ^{32}P-orthophosphate. Similarily, sulfated proteins would be labeled with ^{35}S-sodium sulfate. An important functional category of polypeptides is represented by the DNA binding proteins and these will be separated from the bulk of the infected cell polypeptides using both DNA cellulose chromatography and protein blotting. Those antigens important for the immune response to

the virus in humans would be explored using their interaction with antibodies present in ZIG, a pooled human IgG preparation.

Problems which are likely to be encountered are a possible lack of synchrony in infection owing to the unavailability of high titered cell free virus, the likely failure of the virus to shut down host cell polypeptide synthesis effectively, and the possible co-migration of more than one species of polypeptide on gel electrophoresis.

Accordingly, experiments were initiated with infected cell tissue culture using ^{35}S-methionine to label the polypeptides prior to SDS-PAGE analysis. Figure 12 shows the results of such an experiment using a 9-15% polyacrylamide gradient gel. Nineteen polypeptides were detected in the VZV strain Oka infected cell sample that did not correspond to any in the uninfected cells. Their molecular weights ranged from 175K to 31K. Although the separation appeared good, more viral-specific polypeptides were expected to be seen based on prior reports in the literature. By switching to a straight 12% SDS-PAGE system, better resolution was achieved as seen in Figure 13. In addition, longer gels were used in order to detect as many low molecular weight species as possible. At least 35 polypeptides unique to VZV infected cells were detectable ranging in molecular weight from 240K to 28K. Several different virus strains were compared in Figure 13, VZV strains Scott, OKA and Ellen. The three profiles were essentially identical, with only minor differences noted in the low molecular weight range of 30K-34K. Due to the good overall separation of polypeptides and ease of construction, the remainder of the studies with proteins were carried out with 12% SDS-PAGE as a standard system. In addition, VZV strain OKA was chosen as the major virus strain to analyze since it is currently being

Figure 12. Autoradiogram of ^{35}S-methionine labeled polypeptides of uninfected HFFs (lane M), and VZV strain OKa infected HFFs (lane VZV). Cells were infected with VZV at an "MOI" ofone infected cell to six uninfected cells. At 50-60% CPE, 8 uCi/ml ^{35}S-methionine media was substituted and equal amounts of cells were harvested at 95% CPE (a period of 24 hours), solubilized in disruption buffer, and applied to a 9-15% continuous polyacrylamide gradient gel. In this, and in all subsequent figures involving gel autoradiograms, numbers to the right and/or left of each photograph represent molecular weights in thousands derived from protein markers.


175
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58
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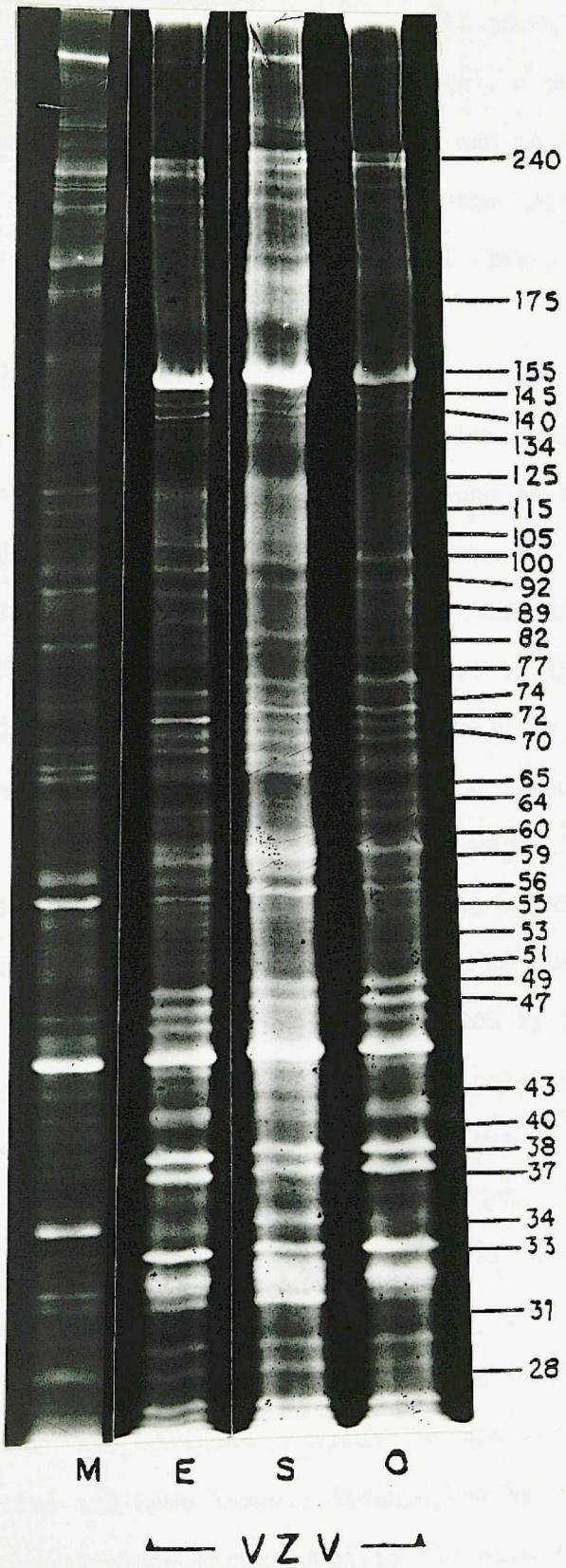
49
43
40
34
33
31
M
VZV

Figure 13. Autoradiogram of ^{35}S-methionione labeled polypeptides of uninfected HFFs and HFFs infected with three different strains of VZV. Lane M, uninfected HFFs; lane E, VZV strain Ellen infected cells; lane S, VZV strain Scott infected cells; lane O, VZV strain Oka infected cells. Cells were infected, labeled, and harvested as in Figure 12 with the exception that a 12% SDS-PAGE system was used instead of a gradient gel.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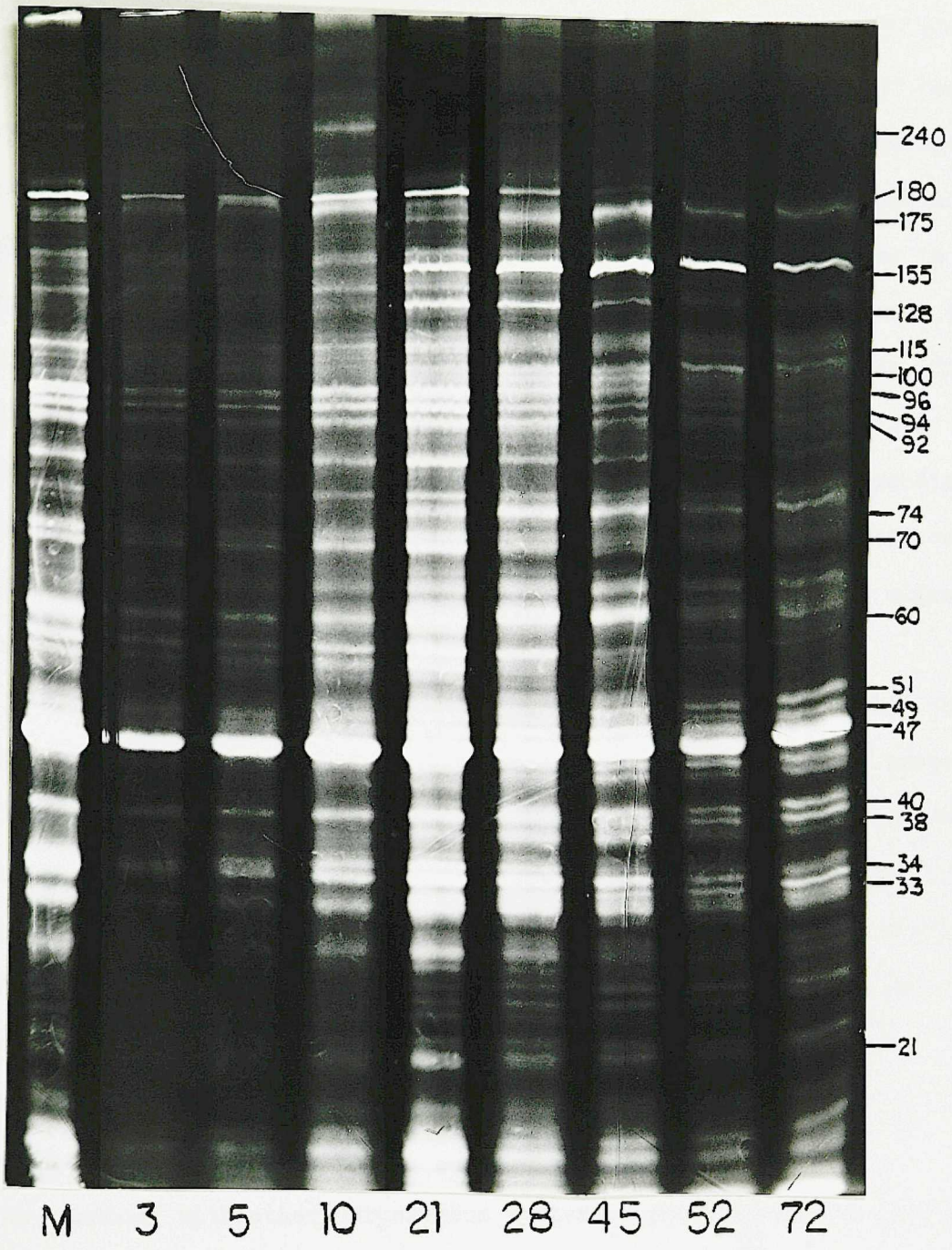
used clinically as a vaccine strain and because it grows well in tissue culture. Based on these data (and others not shown), a catalogue of VZV infected cell-specific polypeptides was obtained and is shown in Table 4 (ICP column). Comparison with other data in the literature shows closest agreement with the results of Takahashi (Table 1), based on molecular weights.

Kinetics of VZV polypeptide production

Now that we had established a pattern of infected cell polypeptides, it became important to attempt to assign functions to these polypeptides. An initial means of approaching assignment of function is to discover at which stage of the infectious cycle various polypeptides are synthesized. Studies were therefore carried out to discern temporal patterns of VZV polypeptide synthesis like those seen in herpes simplex virus infected cells. Figure 14 demonstrates the production of VZV polypeptides over a 72 hour time course. Cells were infected at a slightly higher "MOI" of one infected cell to four uninfected cells in an effort to decrease host cell background as much as possible. 10% CPE was apparent by 21 hours post infection and was 100% by 72 hours.

Although inhibition of synthesis of host cell polypeptides was not as dramatic as that seen with HSV, several patterns do emerge. Many host cell proteins (e.g at molecular weights of 190K, 98K, 71K, 55K, and 35K) cease to be synthesized by 45 hours post inoculation. The first sign of production of new polypeptides in VZV infected cells occurs 10 hours after inoculation when polypeptides at 175K, 130K, 34K, and 21K are visible. All of these polypeptides are synthesized for a short period of time and have largely disappeared by 45 hours after inoculation. These may include virus specific "immediate early" polypep-

Figure 14. Autoradiogram of ^{35}S-methionine labeled polypeptides of VZV infected cells synthesized over a 72 hour time course. Lane M, uninfected HFFs labeled 10-21 hours post mock infection, lane 3, polypeptides produced 0-3 hours post VZV inoculation; lane 5, polypeptides produced 3-5 hours post VZV inoculation; lane 10, polypeptides produced 5-10 hours post VZV inoculation; etc., etc. CPE was apparent at 21 hours post inoculation and was 100% by 52 hours. Samples were prepared as described in materials and methods and run on a 12% SDS-PAGE system.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72

tides. Somewhat later in infection, at 21 hours, a number of other polypeptides appear. Some of these, as in the "immediate early" case, are synthesized only for a short time; polypeptides in that category are the 128K, 60K, 38K, and 35K species. A third set of polypeptides appears either at 21 or 28 hours for the first time and continues to be synthesized until very late in infection. These polypeptides are the 175K, 155K, 115K, 100K, 74K, 65K, 51K, 49K, 40K, and 33K species. This last category of polypeptides may represent the "late" polypeptides of VZV while the previous category may be "early' as opposed to "immediate early". It was difficult to classify the remainder of the infected cell polypeptides because of the possible presence of a host species migrating at a similar molecular weight.

The strong band at 155K was evident at 21 hours post inoculation when CPE was first visible. This polypeptide may be the major capsid protein (Zweerink and Neff, 1981). Our data (see below) confirms this assessment. By 72 hours CPE was 100% and many of the cells had detached from the monolayer. Most of the infected cell proteins (ICPs) seen earlier (Figure 13) are evident in this experiment with the exception of the 240K ICP. Further gels showed that its appearance is spotty and no reason for this behavior is apparent.

Phosphoproteins

By analogy with other herpesvirus systems, phosphoproteins may play an important regulatory role in VZV infections. Thus ^{32}P-orthophosphate labeling was used to identify for the first time the phosphoproteins of VZV. Figure 15 compares the ^{32}P with the ^{35}S-methionine labeled proteins in VZV infected cells. Major virus-specific phosphoproteins are present at 175K and 46K while several more minor bands are seen at 59K,

Figure 15. Autoradiogram of the phosphoproteins of VZV infected and uninfected HFFs. Lanes VZ are the ^{32}P labeled or ^{35}S-methionine labeled polypeptides of VZV infected cells. Lanes M represent an equal number of uninfected cells. Cells were infected with an "MOI" of one infected cell to six uninfected cells. At 50% CPE cells were labeled with ^{32}P-orthophosphate or ^{35}S-methionine and harvested at 90-95% CPE. Equal amounts of uninfected cells were labeled simultaneously.

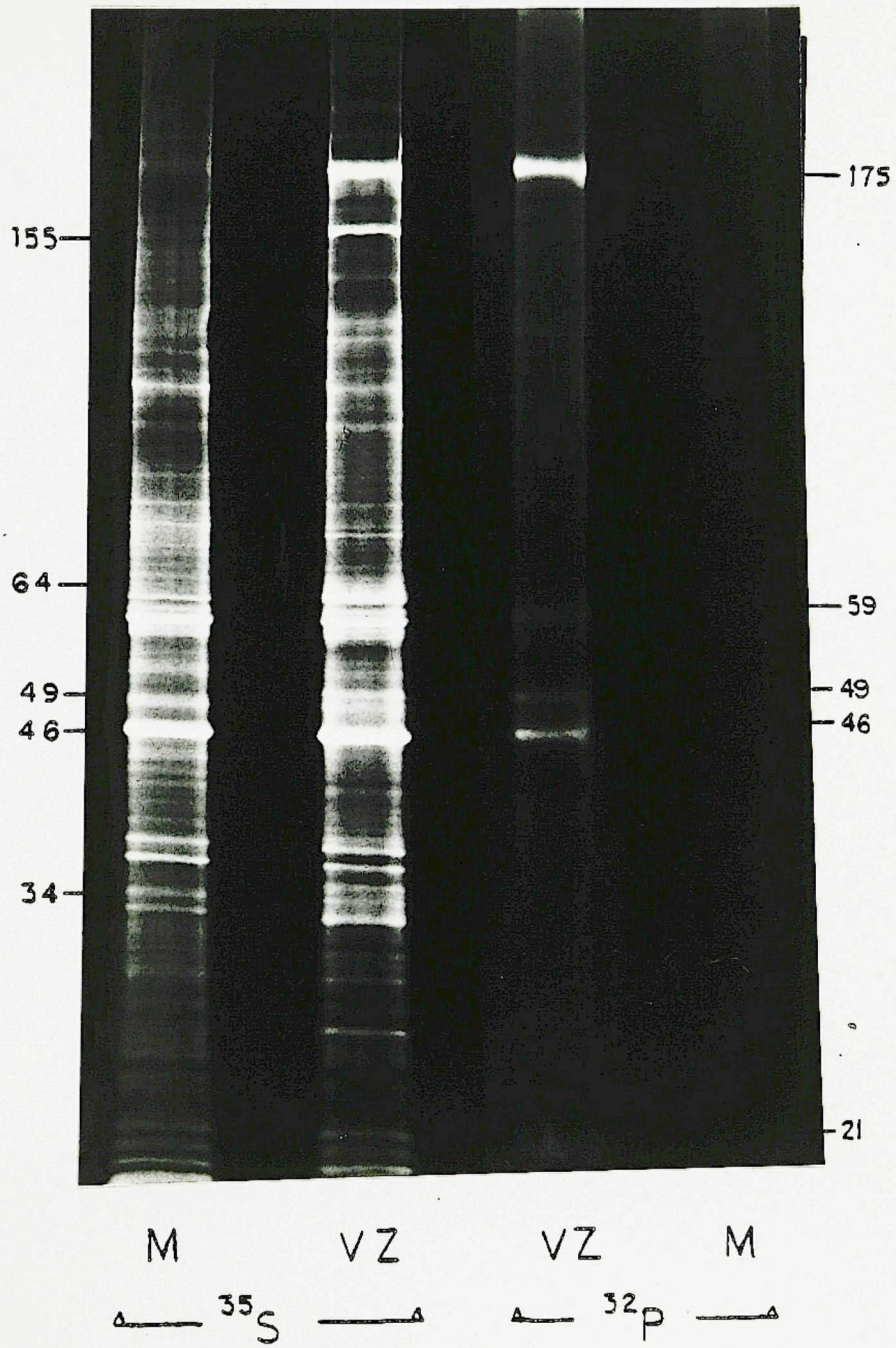
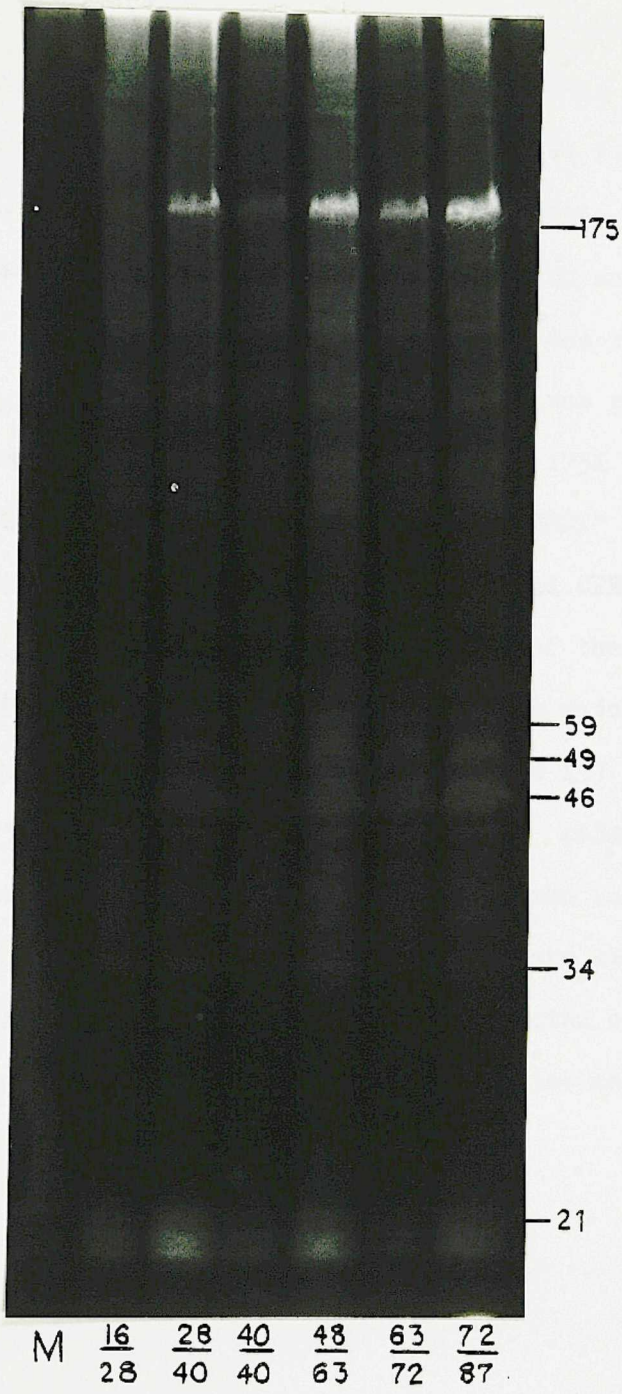

155
64
49
46
34
175
59
49
46
21
M
VZ
VZ
M
35S
32P

Figure 16. Autoradiogram of ^{32}P labeled polypeptides of control and VZV - infected cells over an 87 hour period. Numbers below each lane indicate the labeling period in hours post inoculation. For example, 16/28 equals a labeling period from 16 hours to 28 hours post inoculation with VZV. Uninfected cell lanes (M) were labeled 0-16 hours post mock infection. Equal amounts of cells were prepared for SDS-PAGE analysis as described in the text.


175
59
49
46
34
21
M
16/28
28/40
40/40
48/63
63/72
72/87

49K, and 21K. Surprisingly the uninfected cells showed little or no phosphoprotein labeling. This was consistently seen during all subsequent phosphoprotein studies. The phosphoprotein profile seen in Figure 15 represents species present at late times in infection, when CPE was 90-95%. Since phosphoproteins may constitute an important part of the "immediate early" and "early" population and may undergo phosphorylation - de-phosphorylation cycles during the replicative cycle (Wilcox et al.,1980), a time course experiment was performed and the results are shown in Figure 16. Again the major 175K phosphoprotein is detectable by 28-40 hours post inoculation. The "MOI" in this experiment was one infected cell to six uninfected cells and CPE was not apparent until 48 hours post inoculation. The appearance of the 175K phosphoprotein before CPE and before the appearance of the major capsid protein at 155K as shown in the ^{35}S-methionine (Figure 15) label indicates that this 175K phosphoprotein may be produced relatively early in infection. In addition to the phosphoproteins seen in Figure 15, other minor phosphoproteins at 35K, 21K, and 19K were seen in Figure 16. Thus we have defined a subpopulation of VZV infected cell proteins, the phosphoproteins, which contains two major species and a set of minor ones.

Glycoproteins

From what is known about herpes simplex viruses, glycoproteins would be predicted to be an important subset of VZV polypeptides and have been the subject of a relatively large number of publications (e.g., Asano and Takahashi, 1980; Grose, 1980, Grose et al., 1981; Grose et al., 1983; Takahashi, 1983). It was surprising, therefore, to find that in all the published reports on VZV glycoproteins no experiments had

Figure 17. Autoradiogram of uninfected and VZV-infected cell polypeptides labeled with ^{3}H-glucosamine and ^{3}H-mannose. Lane M, uninfected HFFs; lane H, HSV-1 infected HFFs; lanes O,E and W; VZV strain Oka, Ellen and Webster infected HFFs respectively. The sensitivity of detecting labeled proteins was amplified by the en^{3}Hance procedure (New England Nuclear Corp) prior to drying for fluorography at -60°C.

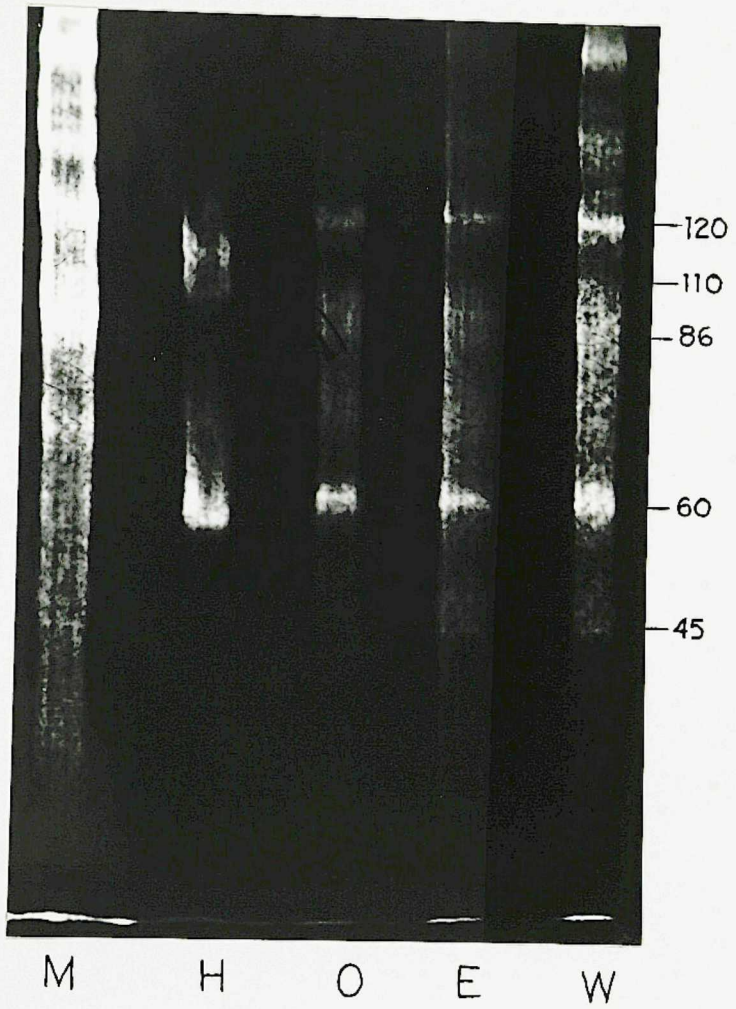
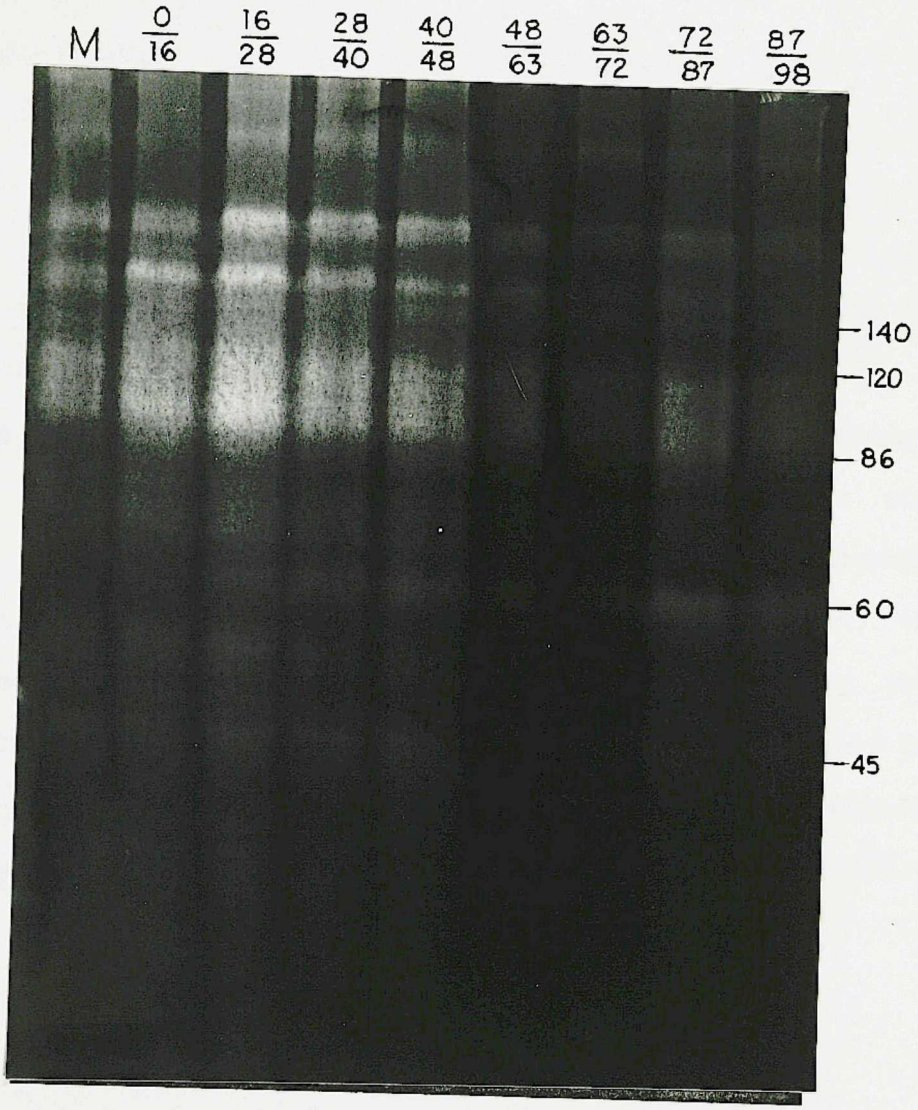

120
110
86
60
45
M
H
O
E
W

Figure 18. Autoradiogram of ^{14}C-glucosamine and ^{14}C-mannose labeled polypeptides of uninfected and VZV-inoculated cells synthesized during a 98 hour period. Lane M represents uninfected HFFs. All other lanes are VZV-inoculated cells labeled for the times indicated after inoculation. The top number indicates the beginning of the labeling period in hours post inoculation and the bottom number indicates the end of the labeling period and time of harvest.

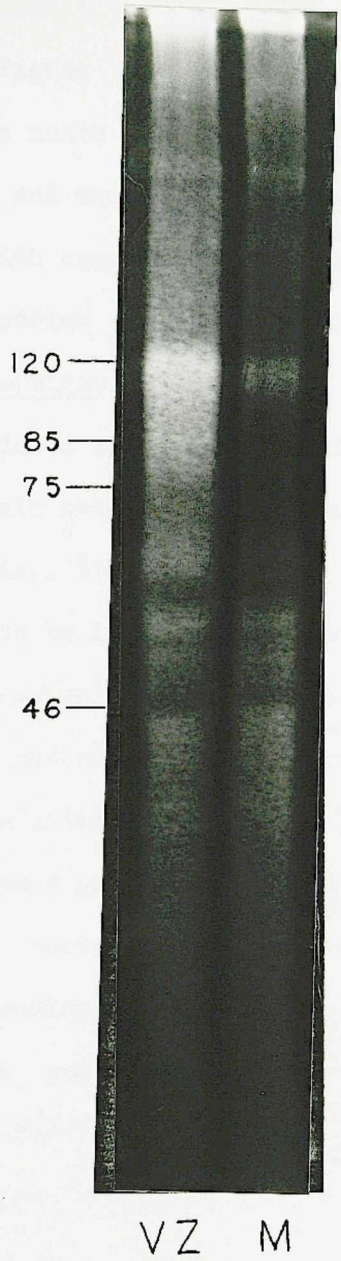

M
0/16
16/28
28/40
40/48
48/63
63/72
72/87
87/98
140
120
86
60
45

been done with more than one labeled sugar. Since glycoproteins are complex entities some of which contain both glucosamine and mannose, the use of only one radiolabeled sugar may result in inaccurate characterization. Accordingly, glycoprotein studies were initiated using a double label with ^{3}H-glucosamine/ ^{3}H-mannose and several strains of VZV infected cells. Figure 17 shows three major zones of glycoproteins at 120K, 110-80K, and 60K. A glycoprotein may also be present at 45K. HSV-1 was used for comparison. However, uninfected cells showed glycoproteins in the same regions of the infected cells except at 60K. The increased amount of label in the VZV strain Webster (lane W) is puzzling since all cells were treated identically. By switching to the more expensive ^{14}C-glucosamine and ^{14}C-mannose labels it was hoped that better resolution would be achieved since direct autoradiography would be possible without enhancement procedures. However, this was proved not to be the case as is shown in Figure 18. Using both ^{14}C labels, a glycoprotein can be seen at 60K by 16-28 hours post infection which coincides with the first appearance of CPE. Unfortunately in any other areas the uninfected profile is very similar. As will be seen later, viral structural glycoproteins can be readily characterized in virion preparations.

Sulfated proteins

In other herpesvirus-infected cell systems, notably HSV infections, a specific subset of polypeptides is known to be sulfated and this has been correlated with function; these are Fc surface receptors (Bauke and Spear, 1979). To investigate the possibility that VZV encodes sulfated glycoproteins, the following experiment was carried out. Radioactive sodium sulfate was used as a label to identify glycopro-

Figure 19. Autoradiogram demonstrating the ^{35}S-labeled sulfated polypeptides of VZV infected cells (VZ) versus the sulfated polypeptides of uninfected cells (M). Dried gels were exposed to XAR-5 film using intensifying screens for 49 days at -60°C.

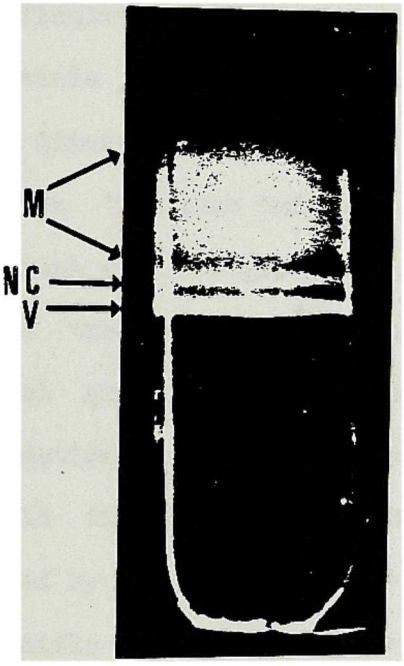

120
85
75
46
VZ M

teins which are also sulfated. In all experiments the labeling was very poor and the resolution on autoradiographs showed smearing typical of glycoproteins. Figure 19 shows the best results obtained with this label after a two month exposure. Two major species can be seen: a band between 100-120K and another at 85K. These areas correspond to glycoproteins seen with sugar labels and suggest, but do not prove, that these sulfated species are glycoproteins.

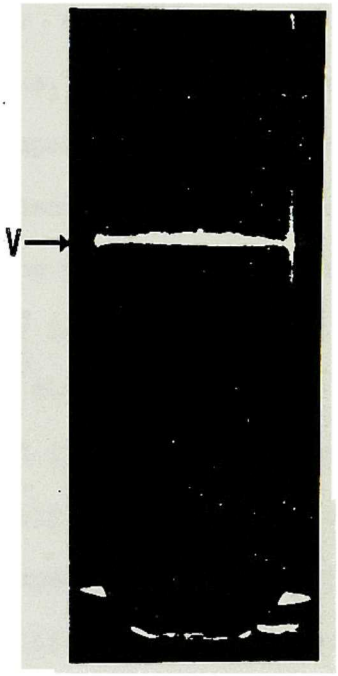
Structural polypeptides

After VZV induced intracellular polypeptides were partially characterized as to their methionine, phosphate, sugar, sulfate content, and rates of synthesis, it was important to identify their functions. The first function to be investigated was which of these polypeptides are structural virus proteins. This investigation required a good procedure for virion and nucleocapsid isolation. Several such techniques are described in the literature and most of these were evaluated experimentally for yield and purity of virion preparation. None of these procedures was deemed adequate. Instead a procedure was developed using sucrose gradients (Dumas et al.,1980) followed by two potassium tartrate/glycerol gradients (Shemer et al., 1980) which resulted in highly purified virions with greatly reduced background of host cell membranes as judged by electron microscopy. Figure 20A shows the bands present after the first potassium tartrate/glycerol gradient. Negative stain electron microscopy of the diffuse upper band (M) revealed disrupted membranous material with only a few viral particles. The middle band (NC) contained some membranous material, but was mostly nucleocapsids with about 10% of all viral particles being enveloped or partially enveloped. The lower band (V) predominately consisted of

Figure 20. Photographs of the first (A) and second (B) potassium tartrate-glycerol gradients used to isolate VZ virions. The V band contained highly purified virions. The NC band contained nucleocapsids, partially enveloped virons, and membranes. The broad M band contained mostly membraneous material and few virions. The V band was reisolated on a second potassium tartrate-glycerol gradient as shown in B.



A



B

enveloped virus with less than 10% nucleocapsids. This band was routinely reisolated on another potassium tartrate/glycerol gradient (Figure 20B) in order to obtain extremely pure virions.

With this isolation technique experiments were performed to distinguish non-structural virus-specific intracellular proteins (ICP) from structural porteins in the virion and nucleocapsid. One of the difficulties in such an assignment is the contamination of virus particle preparations with host cell proteins. None of the reports in the literature has directly addressed this important point. Accordingly, a "pre/post infection" labeling experiment was run by isolating virions from two groups of infected cells. The pre-label group of cells were treated with ^{35}S-methionine before VZV infection and the post-label group of cells were treated with ^{35}S-methionine after viral infection. By comparing the virions purified from the two groups, we could distinguish contaminating host cell polypeptides from those encoded by the virion. Figure 21 shows this comparison. At least 29 virus specific structural polypeptides can be identified. Additional polypeptides may be masked by the intense bands at 115K, 100K, 80K, and 60K. The silver stain of this gel (Figure 22) shows that approximately equal amounts of protein were present in the prelabel and postlabel lanes, while the amount of label is quite different. As a result the amount of host cell protein carryover has been estimated to be less than 1%. The only major protein which we can identify as possible host contamination is at 45K and is presumed to be actin. Some have speculated that actin is not a contaminant but a real part of the virion (Grose and Friedricks, 1982).

Figure 21. Autoradiogram of virions isolated during the pre/post infection labeling experiment. Virions purified from cells labeled with ^{35}S-methionine prior to inoculation (prelabel) were compared to virions purified from cells labeled after inoculation (postlabel). Lane M, uninfected cells; lane VZ, VZV infected cells ; lanes C, VZ-infected cell starting material for virion isolation; lanes V, virions isolated from VZ infected cell starting material.

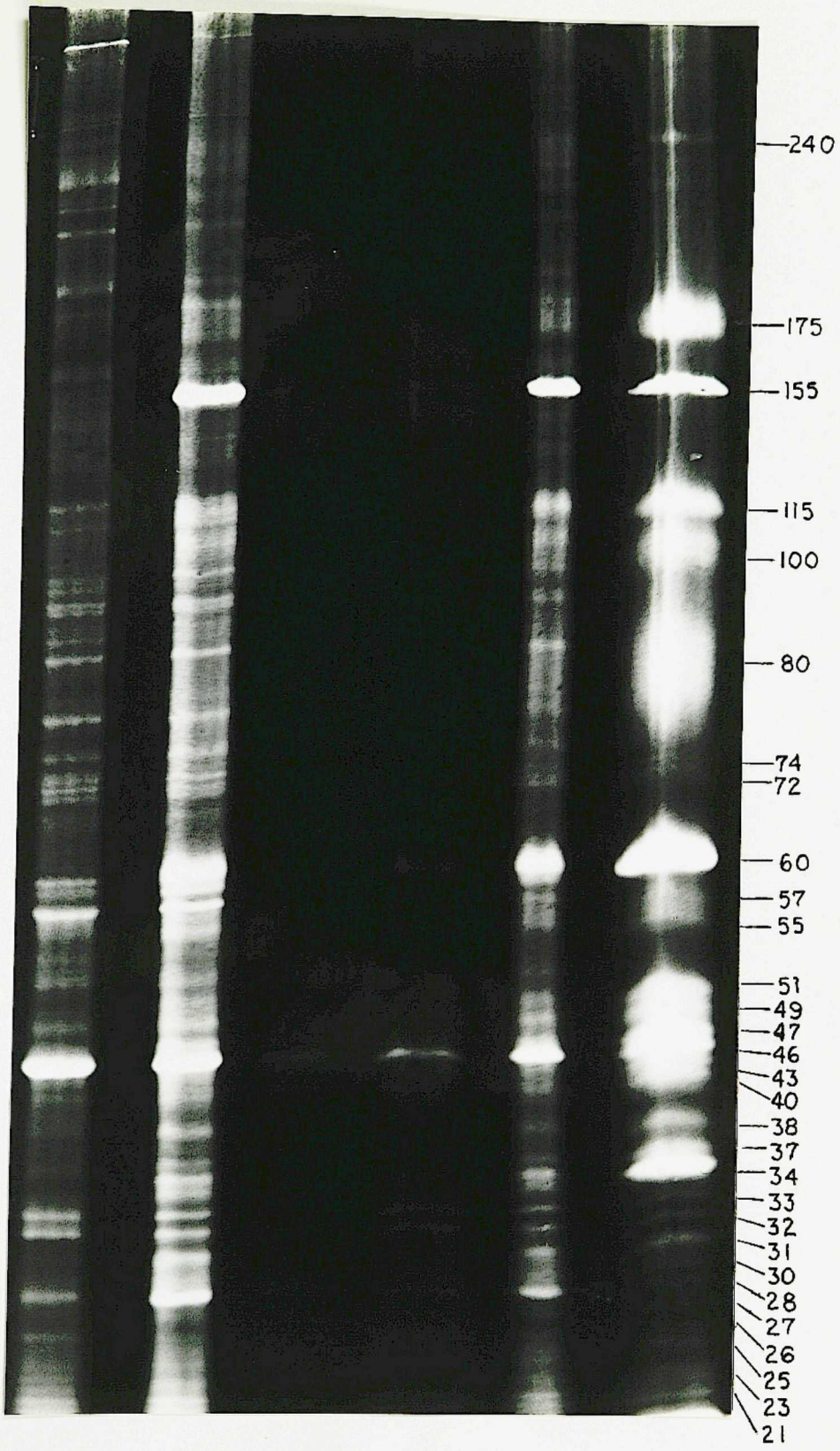
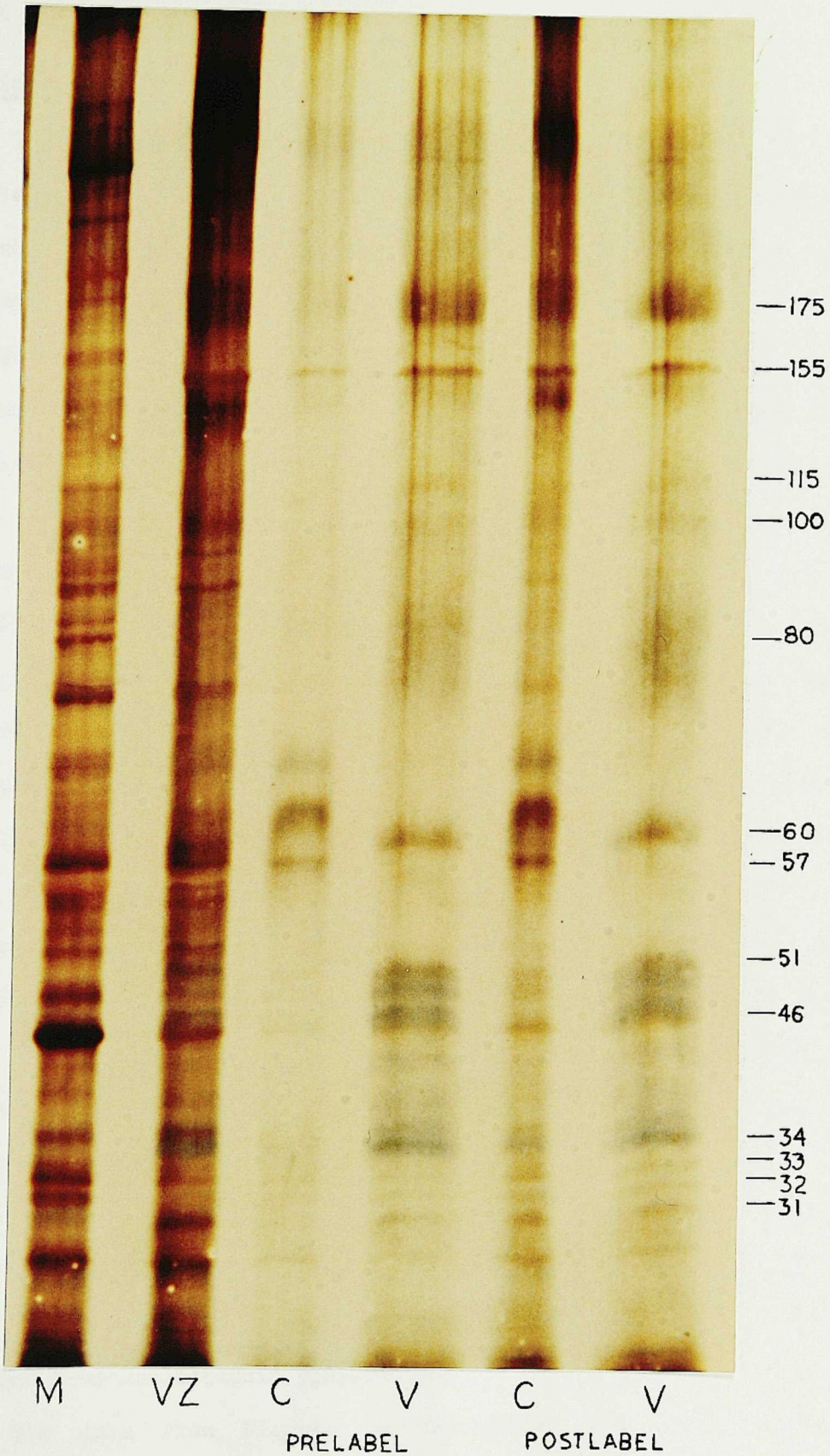

240
175
155
115
100
80
74
72
60
57
55
51
49
47
46
43
40
38
37
34
33
32
31
30
28
27
26
25
23
21
M
VZ
C
V
C
V
PRELABEL
POSTLABEL

Figure 22. Photograph of the gel in Figure 21 after silver staining according to the procedure of Merril et al. (1981). Lanes are identical to those in Figure 21 and the photograph is to scale for ease of comparison.


175
155
115
100
80
60
57
51
46
34
33
32
31
M
VZ
C
V
C
V
PRELABEL
POSTLABEL

Differential radiolabeling of virions

To further characterize the polypeptides of VZ virions, infected cells were labelled with either ^{35}S-methionine, ^{32}P-orthophosphate, ^{14}C-glucosamine/mannose, or ^{35}S-sodium sulfate and the virions isolated as before. Figure 23 shows the comparison of the polypeptides of these virions labeled with the different isotopes. In this analysis the phosphoproteins and glycoproteins can be readily distinguished from the other proteins of VZV. In particular, this allowed a better view of the glycoproteins than had been seen previously (Figures 17 and 18). Five glycoproteins could be detected in the virion at 46K, 60K, 80K, 105K and 115K with the major glycoprotein at 60K. Faint but distinct bands in all the glycoprotein regions may represent different stages of glycosylation. In Figure 24 the glycoprotein patterns of the various steps in the virion isolation procedure are compared to uninfected cells. In this preparation, the "nucleocapsid" bands contained at least 10% enveloped particles. Although the virion preparation nicely identifies the glycoproteins of VZV, faint bands were also seen at 140K, 180K, and 240K . These could be minor virus-specific proteins, or, conceivably, minor host cell components that somehow survived the gradient separations. VZ virions also contain three of the phosphoproteins previously characterized in infected cells (see Figures 15, 16, and 23); while nucleocapsids, prepared by the method of Straus et al. (1981) have five major ^{35}S-labeled proteins at 155K, 115K, 90K, 80K, and 46K (Figure 23). Note that the 175K virion protein is strikingly absent from nucleocapsid preparations.

The data from Figures 14 through 24 have been compiled and summarized in Table 4. For each molecular weight there may exist more

Figure 23. Autoradiogram comparing the polypeptides of purified VZV virions of VZV labeled with ^{35}S-methionine (^{35}VS), ^{32}P-orthophosphate (^{32}VP), ^{14}C-glucosamine/ ^{14}C-mannose (^{14}VC), and ^{35}S-sodium sulfate (^{35}VN). Lane M, uninfected cell polypeptides; lane E, infected cell cytoplasmic extract used to prepare ^{35}S-methionine labeled virons; lane N, ^{35}S-methionine labeled nucleocapsids of VZV. Virions and nucleocapsids were isolated as described in Materials and Methods.

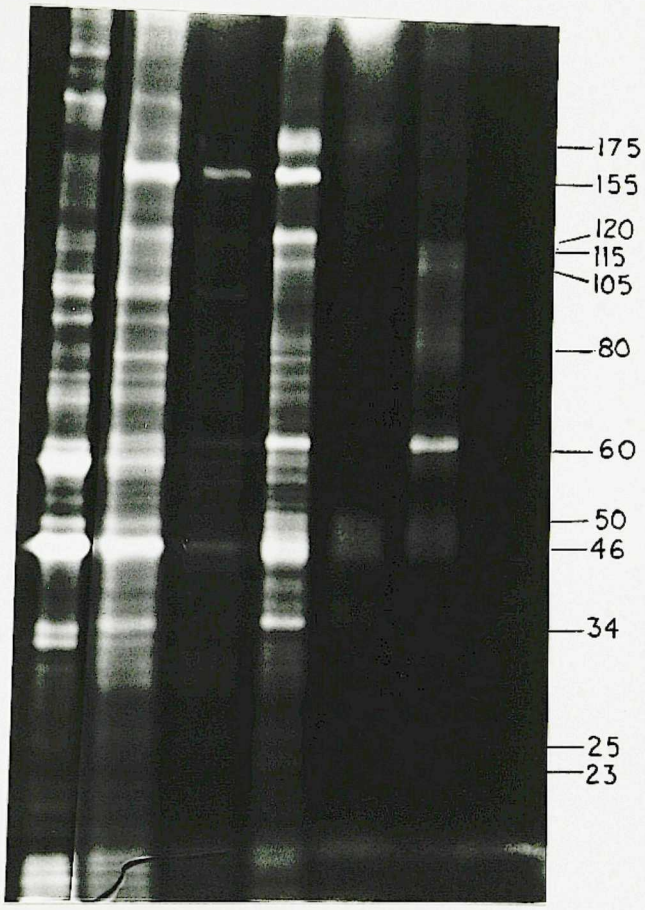

175
155
120
115
105
80
60
50
46
34
25
23
M
E
N
V S
V P
V C
V N

Figure 24. Autoradiogram of ^{14}C-glucosamine and ^{14}C-mannose labeled materials used in the isolation of VZ virions. Lane V, purified VZ virions; lane B, material from the NC band (see Figure 20) of the first potassium tartrate-glycerol gradient; lane N, the nuclear pellet of infected cells used in the virion isolation; lane E, the cytoplasmic extract of infected cells used in the virion isolation; lane U, uninfected cells.

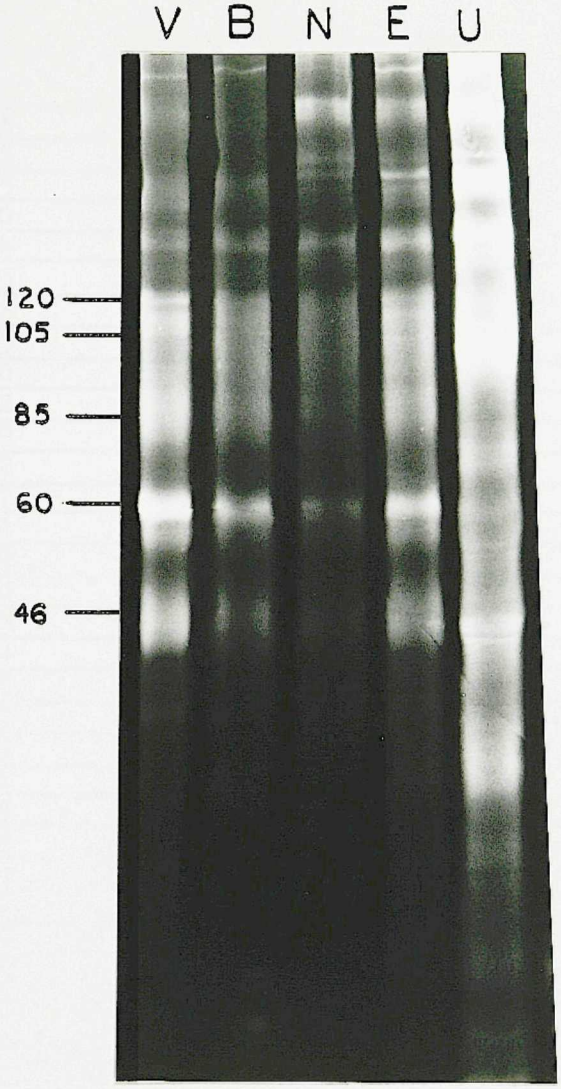

V
B
N
E
U
120
105
85
60
46

Table 4. Preliminary VZV-Specific Polypeptides

Mol.* Wgt.	ICP	Virion	N/C	Phos. Prot.	GP
240	X				
175	X	X		X	
155	X	X	X		
145	X				
140	X				
134	X				
125	X				
115	X	X	X		X
105	X	X			X
100	X	X	X		
92	X				
89	X	X	X		X
82	X	X			X
77	X	X			
74	X	X			
72	X	X			
70	X				
65	X				
64	X	X			X
60	X	X	X		X
57	X	X		X	
55	X	X			
51	X	X			
49	X	X	X	X	
46	X	X	X		X
43	X	X			
40	X	X			
38	X	X			
37	X	X			
34	X	X	X	X	
33	X	X			
32	X	X			
31	X	X			
28	X	X			
27	X	X			
26	X	X			
25	X	X			
23	X	X			
21	X	X			

*Note: The molecular weights are in thousands and for each molecular weight there may be more than one protein. The abbreviations are: ICP, infected cell polypeptides; N/C, nucleocapsid proteins; Phos. Prot., phosphoproteins; GP, glycoproteins.

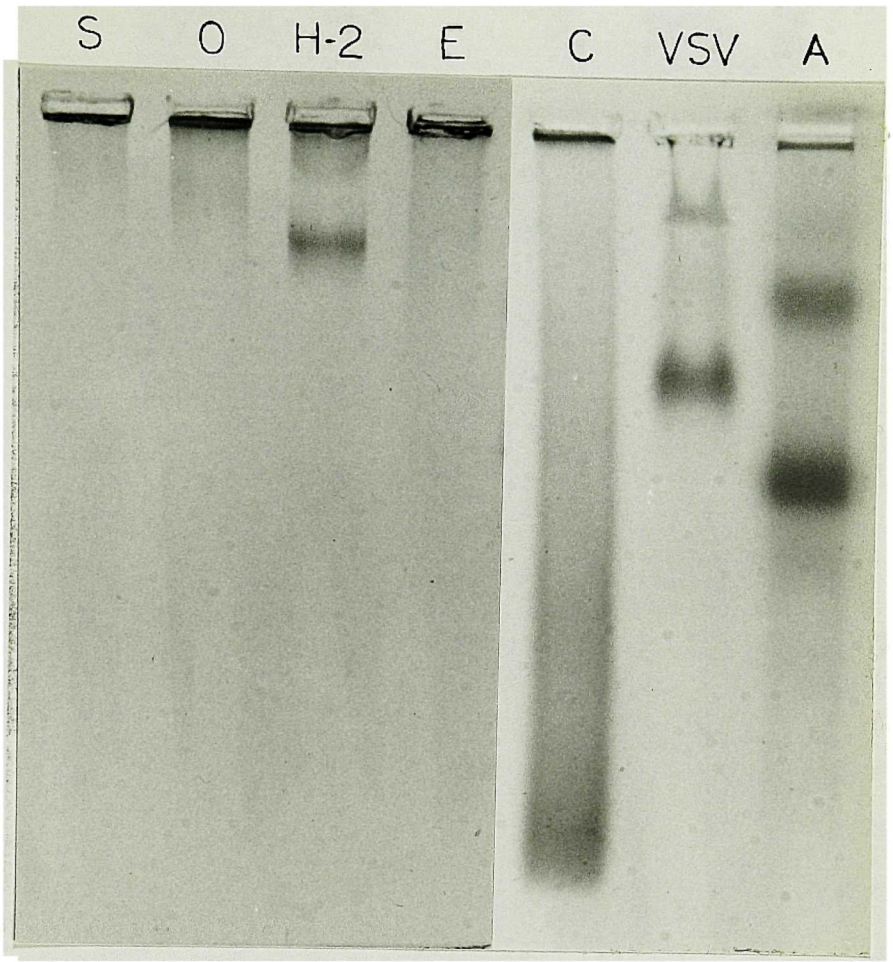
than one polypeptide since the molecular weight of a protein does not necessarily prove polypeptide identity. From this a structural view of VZV polypeptides begins to take shape. For the first time infected cell proteins have been distinguished from virion proteins, nucleocapsid proteins, glycoproteins, and phosphoproteins. From this data specific proteins can now be targeted for individual study in order to develop an understanding of the replication strategy of VZV.

Structural studies on the nucleocapsids of VZV and HSV

As discussed in the introduction to this dissertation, much electron microscopic work has been carried out on the structure of herpesvirus nucleocapsids. This work, carried out chiefly with herpes simplex and equine herpes virus, suggests an icosahedral nucleocapsid structure with 162 capsomeres including 12 pentamers (Wildy et al., 1960; Wildy, 1973). The assumption which is generally accepted is that herpesvirus nucleocapsids are rather similar to each other, but by today's standards the resolution used to develop the model was rather low. In addition, while it has been suggested that VZV is a virus whose poor infectivity is a result of problems with its envelope, no one has addressed the possibility that a specific feature of its nucleocapsid structure could be responsible for its poor performance.

We therefore embarked on a high resolution study of VZV and HSV nucleocapsids. To accomplish this, nucleocapsid preparations of high purity were required. A technique using agarose gel electrophoresis developed by Dr. P. Serwer (1978) and used successfully in high resolution EM of bacteriophage T7 was tried with herpesviruses in order to isolate pure nucleocapsids simply and rapidly. Figure 25 shows that such electrophoresis is possible with HSV-2 , VSV, and adenovirus but

Figure 25. Agarose gel electrophoresis of VZV strain Scott (S), VZV strain Oka (O), HSV-2 (H-2), VZV strain Ellen (E), coronavirus MHV-59 (C), vesicular stomatitis virus (VSV), and adenovirus (A). Gels were constructed with 0.9% low melt agarose in a horizontal mini-gel apparatus and stained with Coomassie Blue.


S
O
H-2
E
C
VSV
A

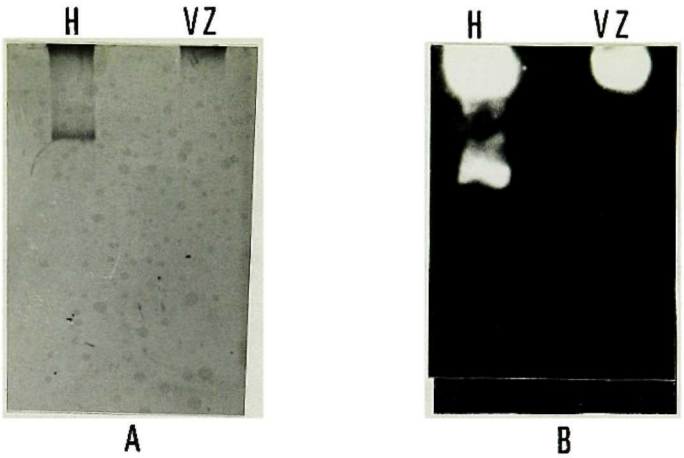


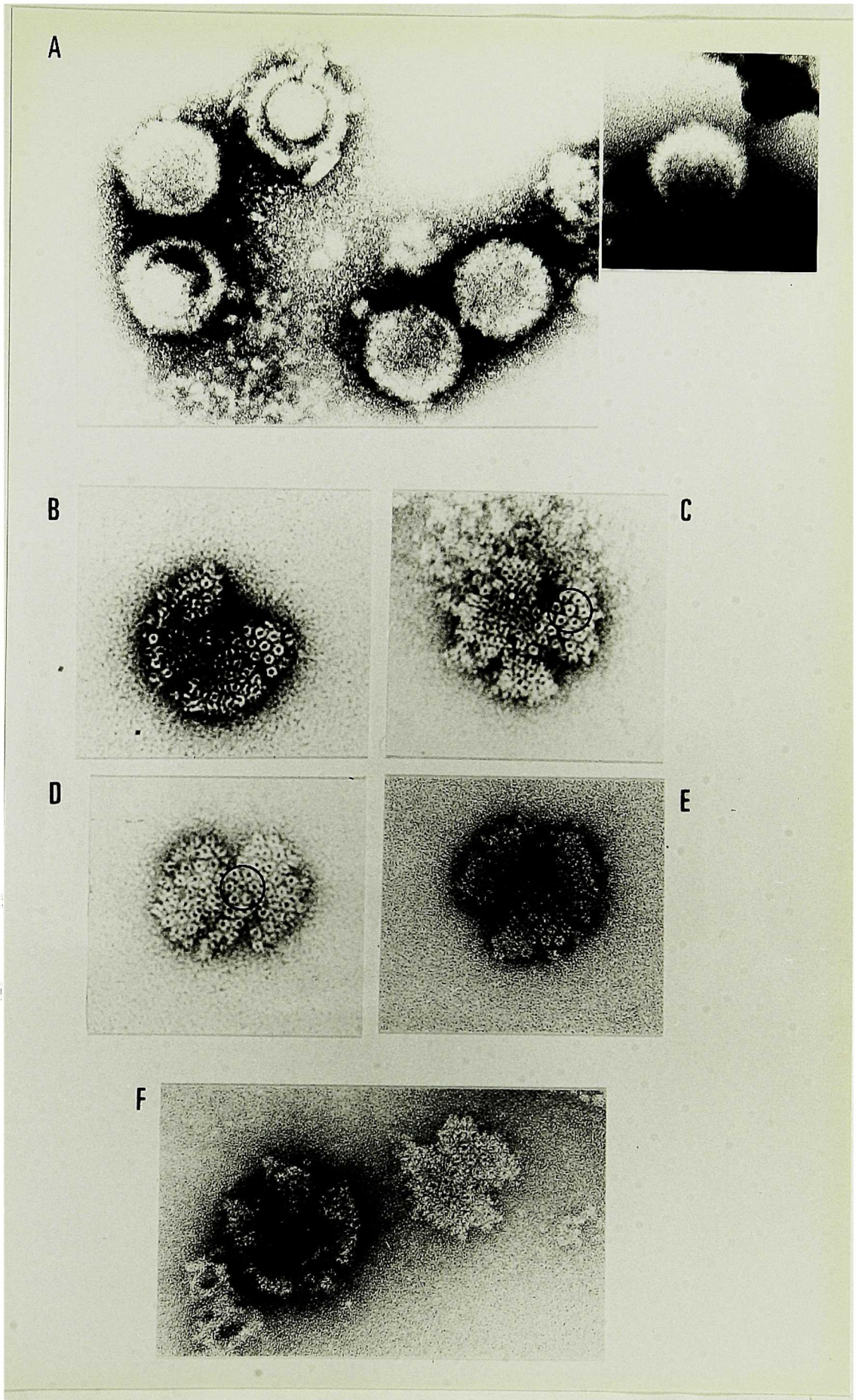
Figure 26. Autoradiogram (B) of the nucleocapsids of HSV-2 (H) and VZV (VZ) after agarose gel electrophoresis (A). The gel was constructed as described in Materials and Methods and photographed after Coomassie Blue staining. The gel was dried under heat and vacuum and exposed to XAR-5 film. Nucleocapsids were labeled with ^{35}S-methionine.

unfortunately not with VZV. Using radiolabeled nucleocapsids, it was evident that VZV remained mainly in the sample well while HSV-2 migrated into the gel (Figure 26). This suggests that although the two nucleocapsids (HSV and VZV) look alike at a gross level their electrophoretic properties are different. Therefore, they may have some important structural differences.

Attempts to extract nucleocapsids from the gel by passive diffusion were unsuccessful as were attempts at thin sectioning the HSV-2 band for EM. The nucleocapsids that were seen were misshapen and too few in number to be of value. Therefore, we returned to the standard nucleocapsid preparation of Straus et al. (1981) as the optimal technique to obtain samples for further structural work (see Figure 27A). This preparation yielded large numbers of nucleocapsids which showed icosahedral shapes in which capsomeres could be visualized. The symetrical array of the capsomeres could also be seen. On occasion a unique phenomenon was observed. The nucleocapsid, for reasons unknown, would split open and lie flat in a "pac-man" or "maltese-cross" configuration and the hexagonal packaging of the capsomeres became apparent (Figure 27B-F, circles).

In order to better visualize the capsomeres, a flattened surface preparation of capsids was sought which would allow better resolution and eliminate the problems of focusing on superimposed images in an icosahedron. Several dissociation and reassembly techniques that were successful with T-even phage capsids were tried with VZV and HSV-2. It was hoped that large sheets or rafts of capsomeres would form so that computer image enhancement would be accomplished more easily. Various chemical disruption-reassociation techniques which included 6M guan-

Figure 27. Electron micrographs of HSV-2 nucleocapsids isolated from density gradients of infected cells. The typical appearance of nucleocapsids is shown in A. Other "pac-man" and " maltese-cross" like structures are occasionally seen as nucleocapsids, for reasons unknown, fall apart (B-F). Circles indicate hexagonal packaging of the capsomeres. Specimens were negatively stained with uranyl acetate. Magnification: X 150,000.


A
B
C
D
E
F

idium hydrochloride, 8 M urea and citraconic anhydride (Wurtz et al., 1976; Aebi et al., 1977) all proved unsuccessful. However, mild trypsin treatment, as first reported by Palmer et al.(1975), produced some interesting results. Equal amounts of ^{35}S-methionine labelled nucleocapsids were treated with 9 units of trypsin for various times and then subjected to SDS-PAGE analysis. Figures 28 and 29 illustrate dramatically different cleavage patterns between VZV and HSV-2. By thirty minutes the VZV nucleocapsid showed 8 major bands at 155K, 116K, 93K, 58K, 50K, 46K, 43K, and 36K. Although the major capsid protein band (155K) is still present at 30 min, its intensity was decreased. In contrast, after six minutes, HSV-2 yielded 4 major bands at 85K, 82 K, 52K, and 48K. The 155K major capsid protein was greatly reduced by this time, which indicated that the HSV-2 nucleocapsid has a greater sensitivity to trypsin than the VZV nucleocapsid. By 30 min the 52K fragment is also reduced in intensity while a 38K fragment is increased in intensity. This difference in trypsin cleavage suggests, as did the agarose gel electrophoresis, that the capsid proteins of these two herpesviruses may assemble into structures that look similar in the electron microscope but are very different at the molecular level.

The trypsin treatment also provided some interesting views of disrupted nucleocapsids in the electron microscope. Using uranyl acetate as a negative stain because of its superior performance, several intriguing views of the flattened, trypsin treated capsids, with doughnut shaped capsomeres of HSV-2, were observed. Treatment with trypsin for two min produced fragments (Figure 30) in which the 155K polypeptide was still intact. Of interest in these micrographs is the

Figure 28. Autoradiogram demonstrating the effects of trypsin on the nucleocapsid polypeptides of HSV-2. Lane H represents the ^{35}S-methionine labeled nucleocapsid preparation of HSV-2 without trypsin. Lanes 0-30 represent HSV-2 nucleocapsids subjected to 9 units of trypsin activity for the time (in minutes) indicated.

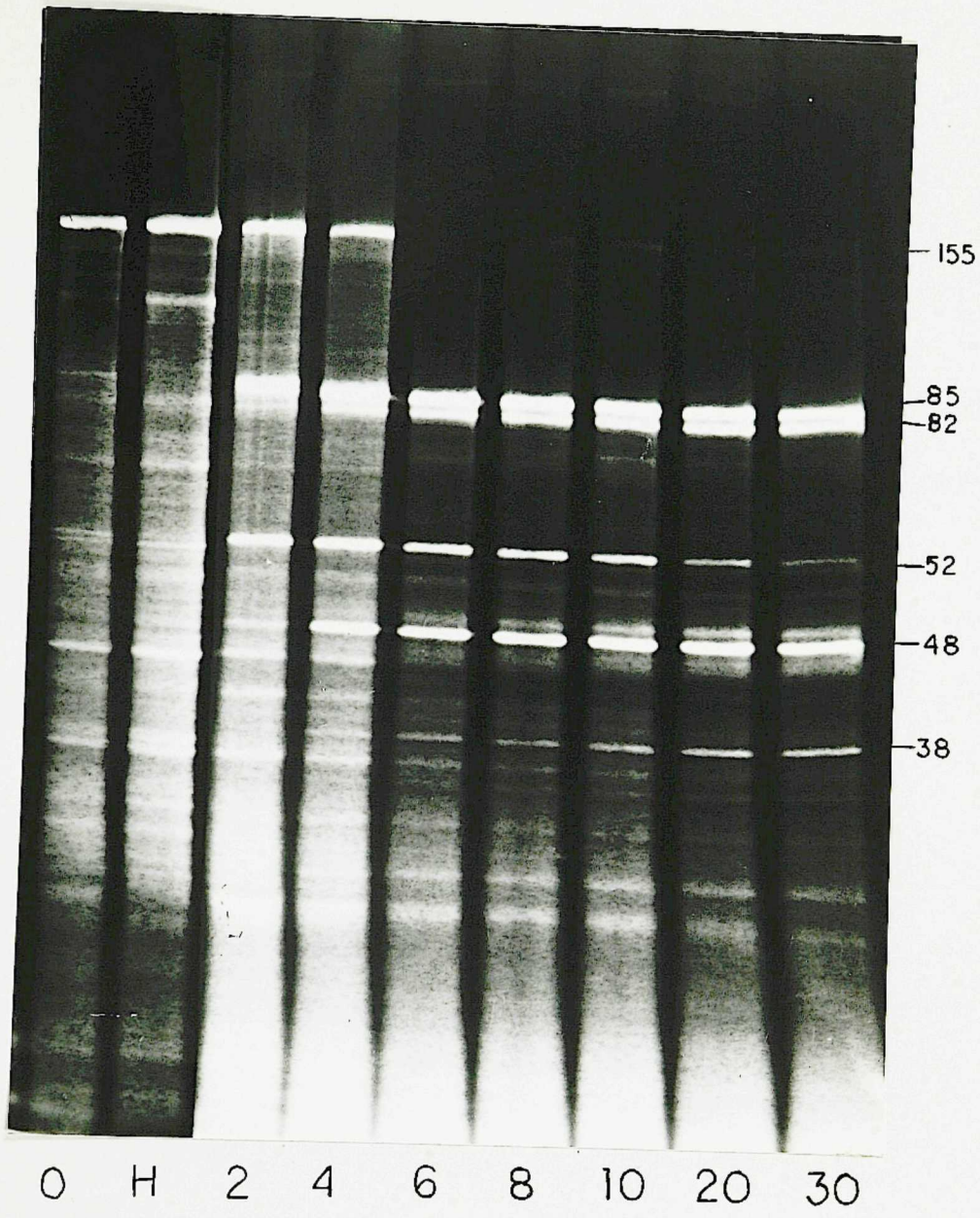

155
85
82
52
48
38
0 H 2 4 6 8 10 20 30

Figure 29. Autoradiogram demonstrating the effects of trypsin on the nucleocapsid polypeptides of VZV. Lane VZ represents the ^{35}S-methionine labeled nucleocapsid preparation of VZV without trypsin. Lanes 0-30 represent VZ nucleocapsids subjected to 9 units of trypsin activity for the time (in minutes) indicated.

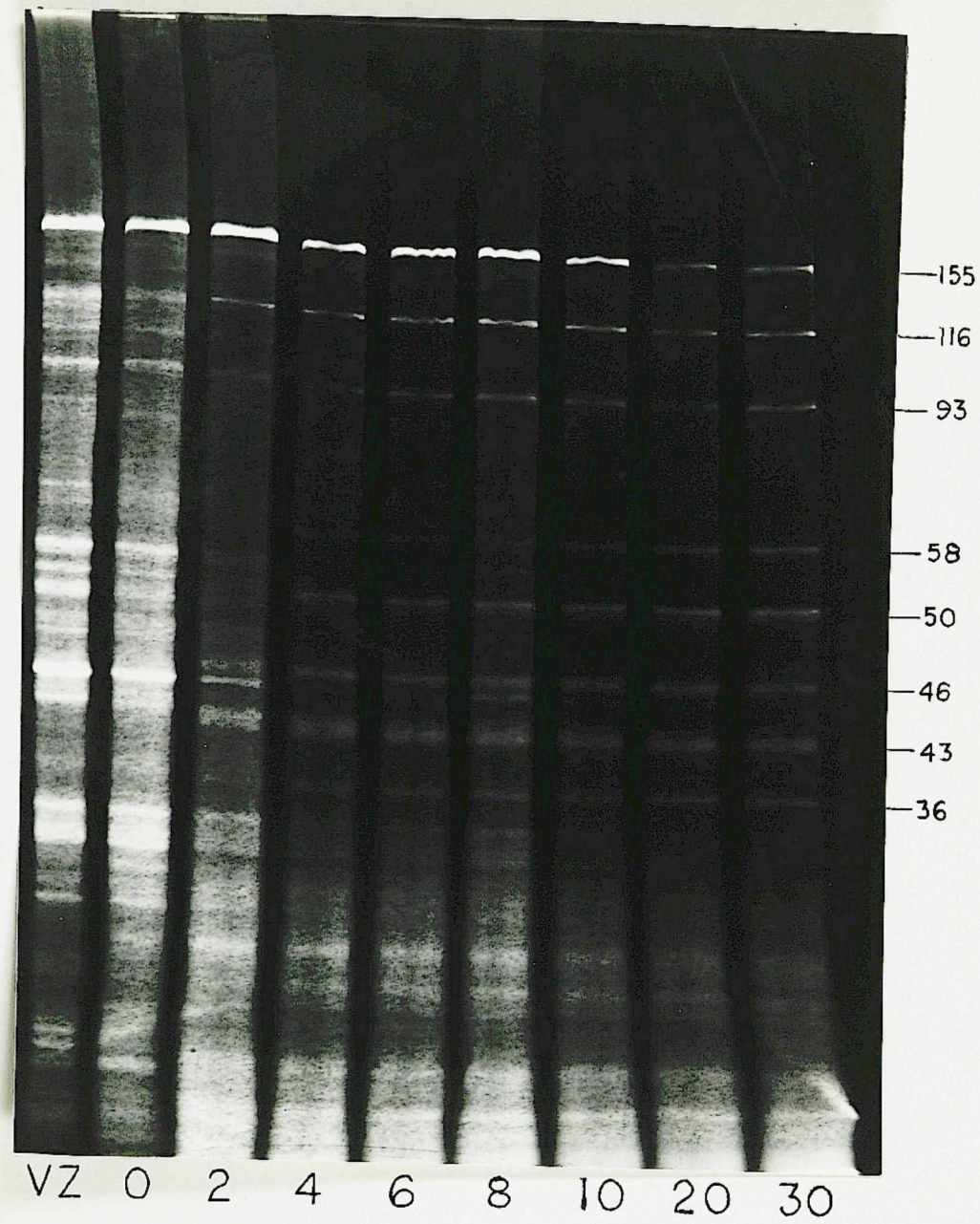

155
116
93
58
50
46
43
36
VZ
0
2
4
6
8
10
20
30

Figure 30. Electron micrographs of HSV-2 nucleocapsids treated with trypsin as described in materials and Methods. After two min trypsin activity was inhibited with aprotinin and samples were negatively stained with uranyl acetate. Several fragments of nucleocapsid were present with the vertex capsomeres (pentons) missing (arrows). Magnification: X 150,000.

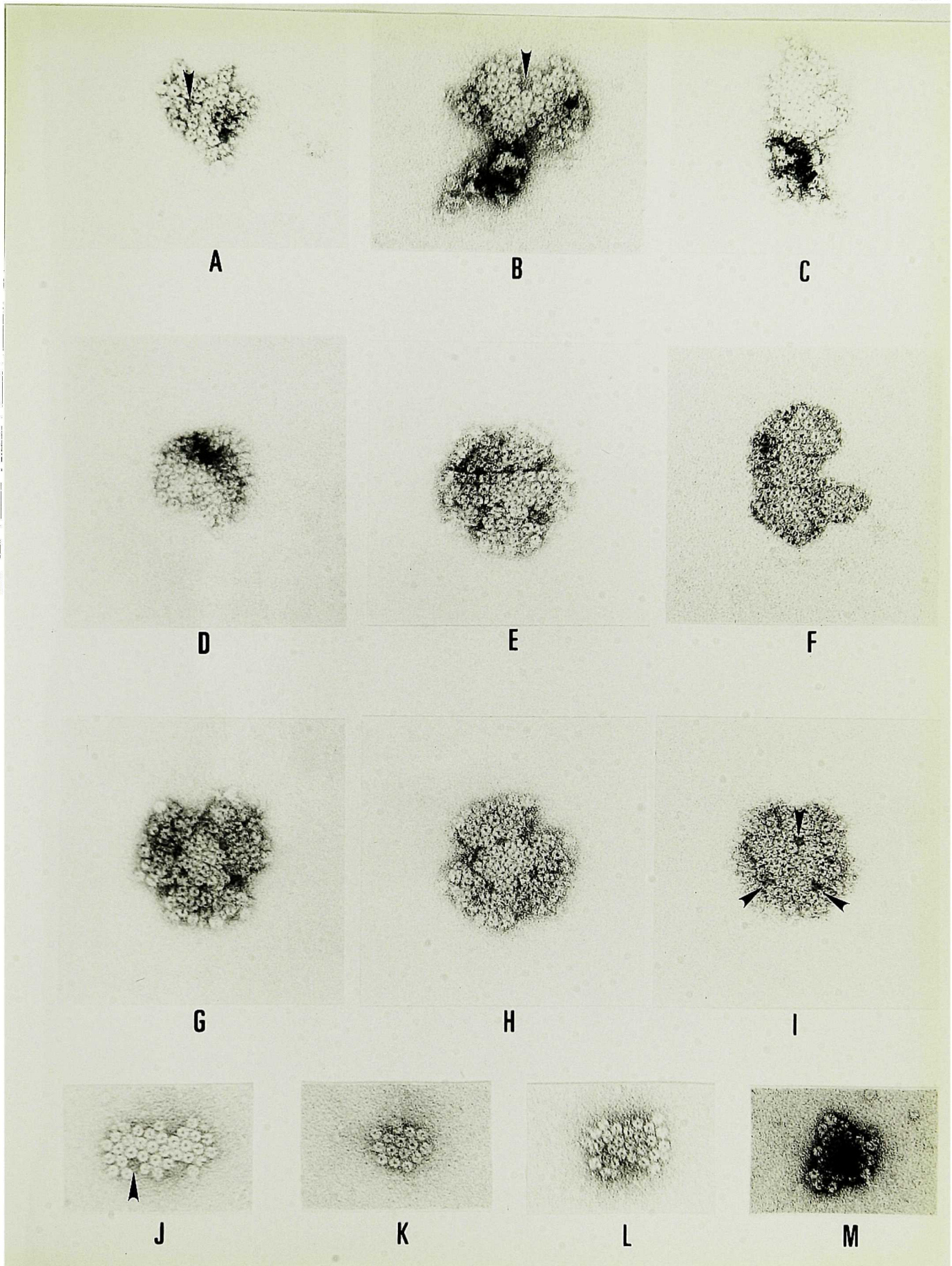
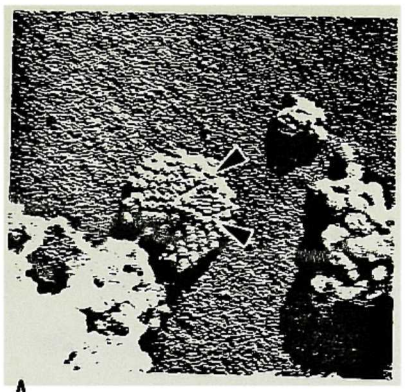
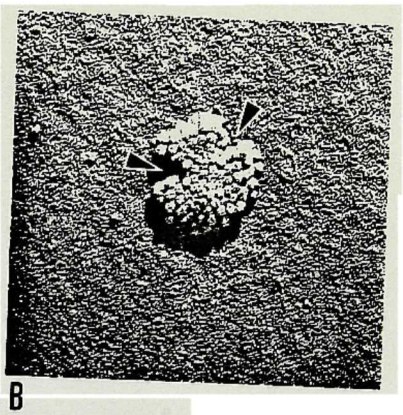

A
B
C
D
E
F
G
H
I
J
K
L
M

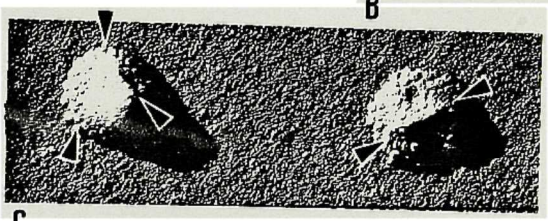
Figure 31. Cryo-electron microscopy of HSV-2 nucleocapsids using the freeze drying and shadowing method of Kistler et al. (1977). In A-C the axes of symmetry of the nucleocapsid are apparent (arrows). In D the nucleocapsid appears to be split with the core still attached (arrow). In F and G arrows indicate smooth surfaces that may represent the inner surface of the nucleocapsid. Magnification: X 100,000.



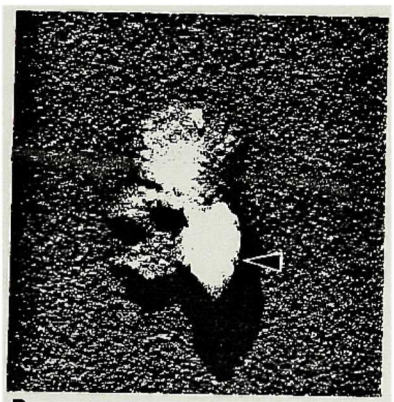
A



B

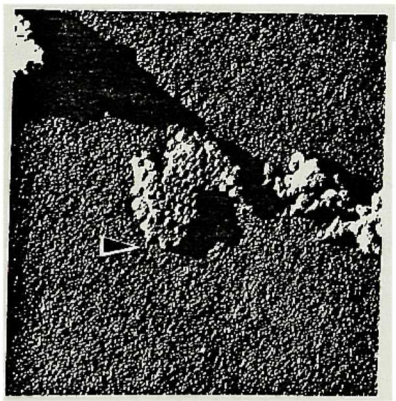


C

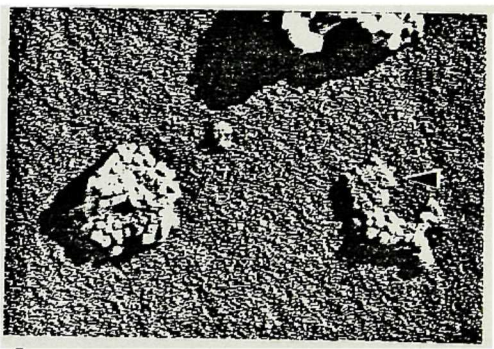


D

E



F



G

apparent susceptibility to trypsin of the penton capsomere (pentamer). It appears to have fallen out (e.g. arrows at Figure 30A, B, and J) and somehow helped create these peculiar structures.

Because trypsin may alter the structure and organization of the capsid proteins, an alternate technique was also used. Figure 31 shows HSV-2 nucleocapsids after freeze-drying and platinum shadowing as described by Kistler et al. (1977). Twofold axes of symmetry are apparent in A-C and hexagonal packaging of the capsomeres can be seen in E. In D the nucleocapsid appears to be split in half with the core still attached and in F-G a smooth surface was detected that may be the inner nucleocapsid surface (arrows). This technique has proved promising since it is relatively easy to perform and results in high quality photographs suitable for image enhancement. Computer assisted image enhancement of the images in Figures 30 and 31, should permit evaluation of the subunits of the pentons and hexons.

The typical VZV nucleocapsid resembled those of HSV. On occasion unique aspects of the core structure were seen in the nucleocapsids (Figure 32A-D). The core appears to be tightly wound filament which is sometimes seen to be attached to the inner surface of the capsid (Figure 32A).

"Pac-man" and "maltese-cross" like structures were also seen (Figure 33) and looked similar to those of HSV-2. Hexagonal packaging of the capsomeres was seen (circles, Figure 33A ,B and D). Unfortunately trypsin disruption of VZV did not produce any fragments like those seen with HSV-2 (Figure 30). This may indicate a structural difference between VZV and HSV nucleocapsids.

Further ultrastructural studies on the nucleocapsids of HSV-2 and

Figure 32. Electron micrographs of VZV nucleocapsids. Several views of the core structure are seen in A-D. The usual appearance of nucleocapsids is seen in E. Specimens were negatively stained with uranyl acetate. Magnification: X 150,000.

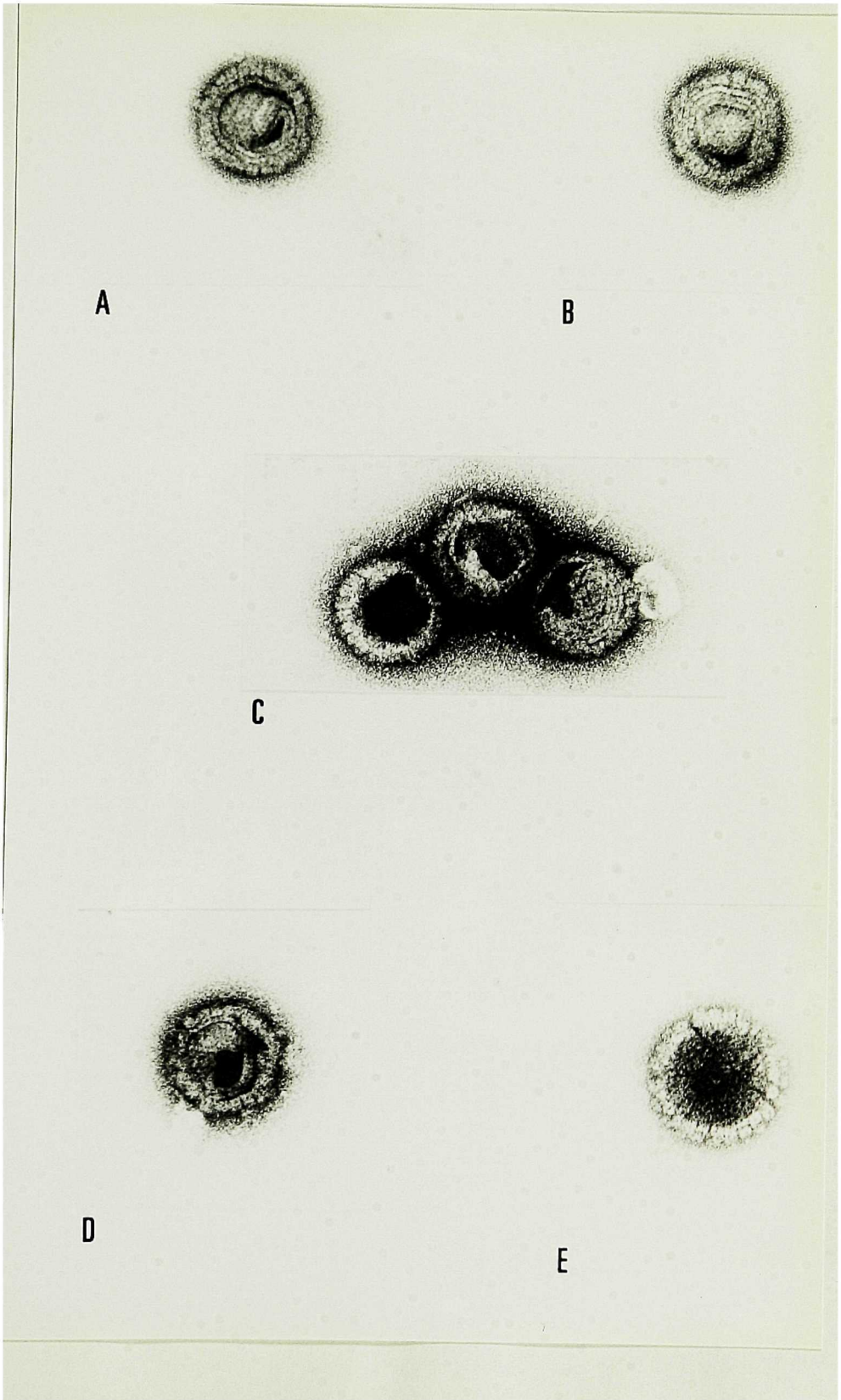
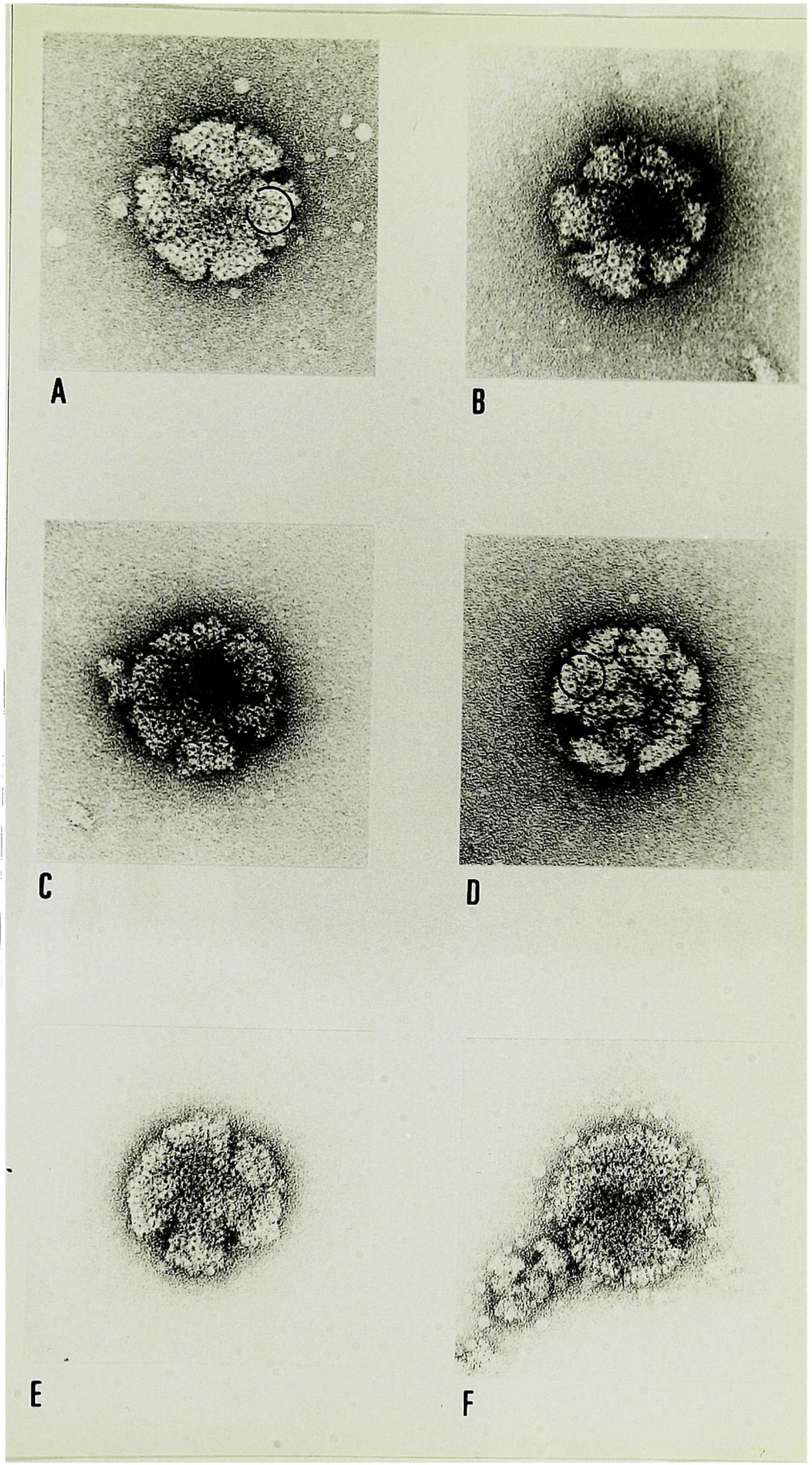

A
B
C
D
E

Figure 33. Electron micrographs of unusual VZV nucleocapsids. "Pac-man" and "maltese-cross" like structures were occasionally seen in negatively stained nucleocapsid preparations (A-F). Circles indicate hexagonal packaging of capsomeres. Magnification: X 150,000.


A
B
C
D
E
F

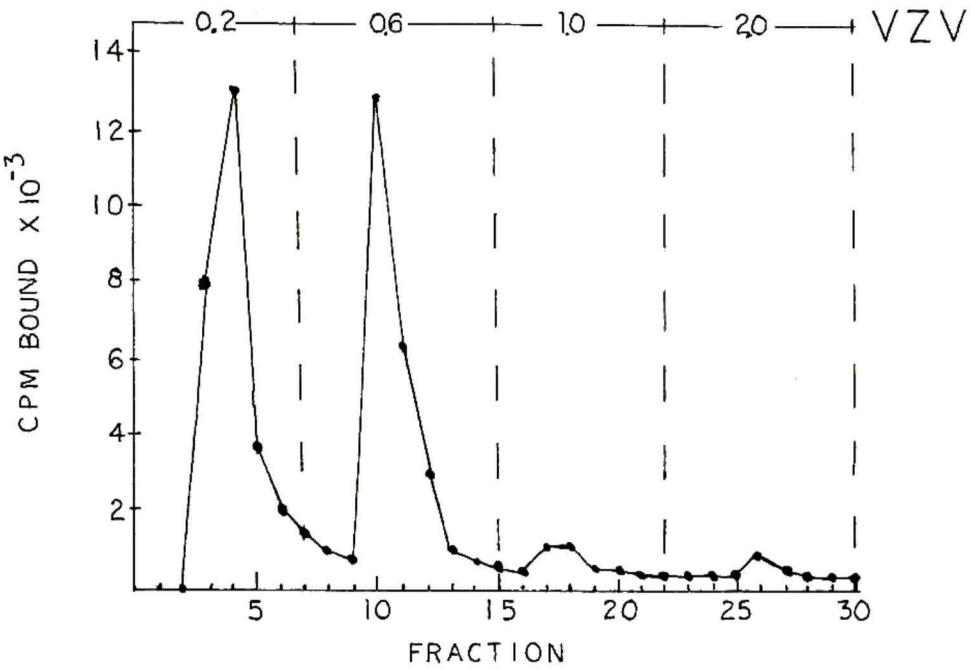
VZV are in progress and the freeze-drying and shadowing technique is being refined in a collaborative study with Dr. A. Steven, Laboratory of Physical Biology, NIH. Bethesda, MD. We now have a sufficiently large number of HSV-2 preparations to start computer analysis, and, while that is in progress, more VZV images will be generated so that the comparison between the two capsids can be made.

DNA cellulose chromatography

It was also important to investigate a second group of proteins which could be assigned a specific function. These are the DNA binding proteins, which have been extensively studied in HSV but not at all in VZV. HSV DNA binding proteins have been shown to include enzymes, controlling proteins, and structural proteins which are frequently complexed with DNA (Bayliss et al., 1975; Purifoy and Powell, 1976; Hay, 1979).

Using single-stranded and double-stranded DNA cellulose column chromatography, ^{35}S- labeled proteins from uninfected and VZV infected cells were eluted with steps of 0.2 M, 0.6 M, 1.0 M and 2.0 M NaCl in buffer A as described in Materials and Methods. The elution profiles of infected and uninfected extracts from both single and double-stranded DNA cellulose columns show significant differences. Specifically, the 0.6 M fractions from the columns with infected cell extracts are greatly enriched in labeled material as compared to 0.6 M fractions from columns with uninfected cell extracts (Figures 34 and 35). Enrichment is also seen in the 1.0 M and 2.0M fractions. Thus, based on the column elution profiles, DNA binding proteins in VZV infected cells have, as a group, a higher affinity for DNA than do the majority of DNA binding proteins in host cells.

Figure 34. Elution profiles of infected (VZV) and uninfected (U) extracts from single stranded DNA cellulose columns. Counts (^{35}S- methionine label) represent a 50 ul aliquot from 2.5 ml fractions. Proteins from peak fractions can be seen in Figures 36 and 37.


0.2
0.6
1.0
2.0
VZV
CPM BOUND X 10^{-3}
14
12
10
8
6
4
2
5
10
15
20
25
30
FRACTION

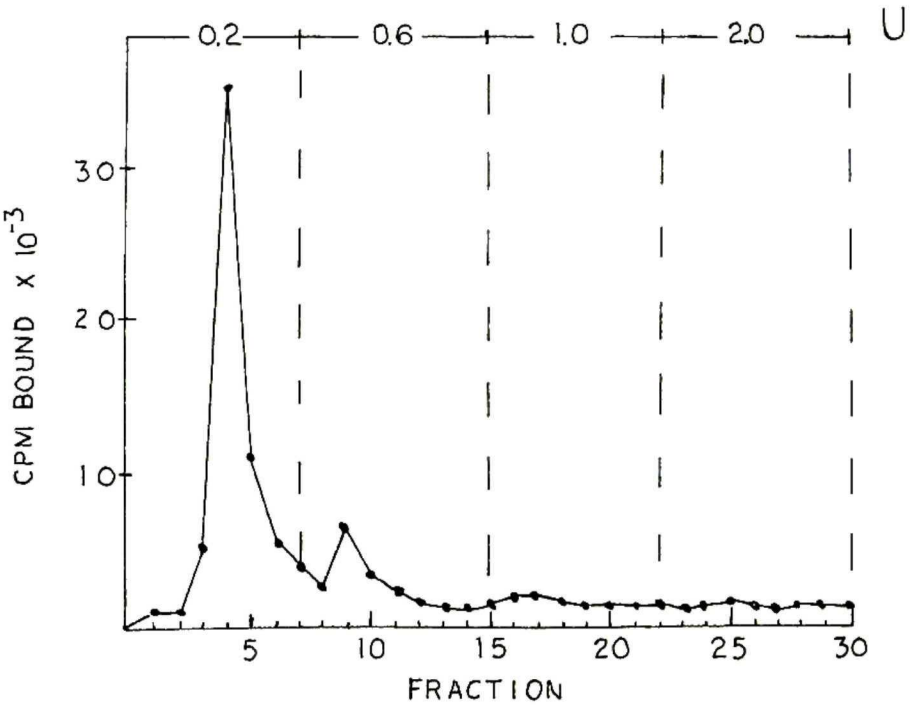
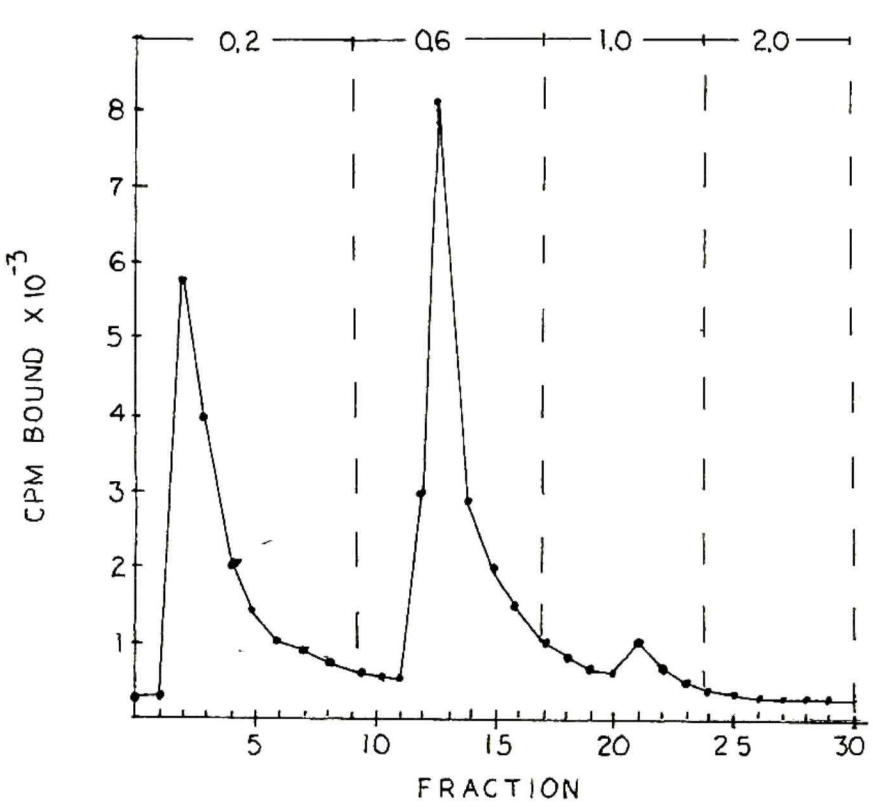

0.2
0.6
1.0
2.0
U
CPM BOUND X 10^{-3}
30
20
10
5
10
15
20
25
30
FRACTION

Figure 35. Elution profiles of infected (VZV) and uninfected (U) extracts from double stranded DNA cellulose columns. Counts (^{35}S-methionine label) represent a 50 ul aliquot from 2.5 ml fractions. Proteins from peak fractions can be seen in Figures 36 and 37.


VZV
0.2
0.6
1.0
2.0
CPM BOUND X 10^-3
FRACTION

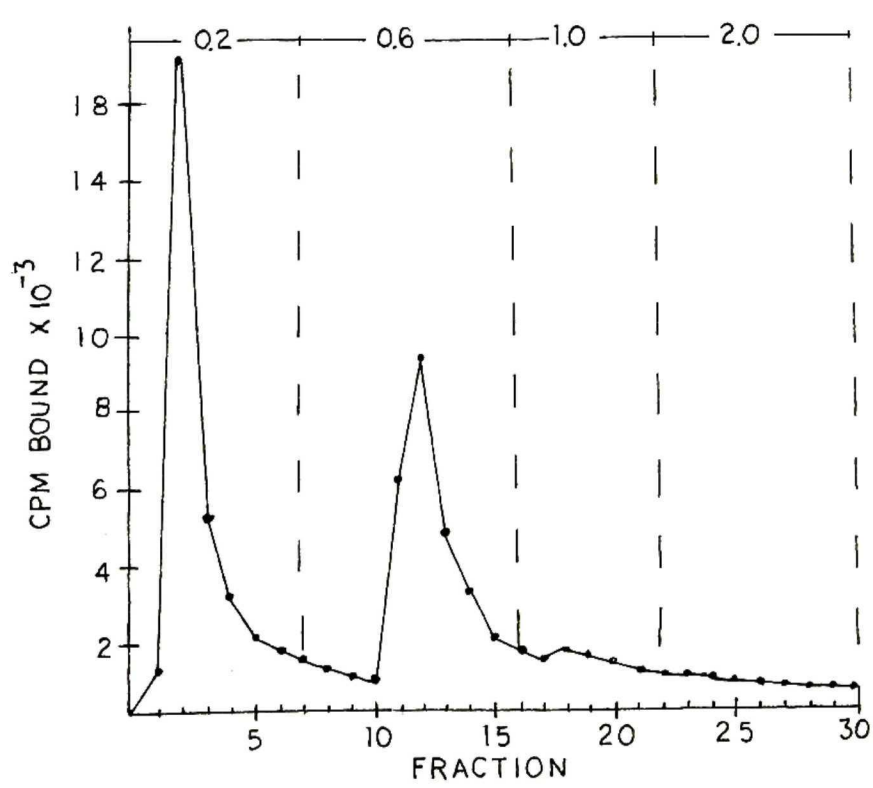

U
0.2
0.6
1.0
2.0
CPM BOUND X 10^-3
FRACTION

Figure 36. Autoradiogram of the uninfected cell (HFF) DNA binding proteins. 0.2M, 0.6M, 1M, and 2M represent the acetone precipitated proteins eluted from single stranded (left) and double stranded (right) calf thymus DNA cellulose columns with increasing NaCl concentrations. Lane VZ is the VZV-infected cell extract used to identify VZV DNA binding proteins. Molecular weight standards are listed on the right.

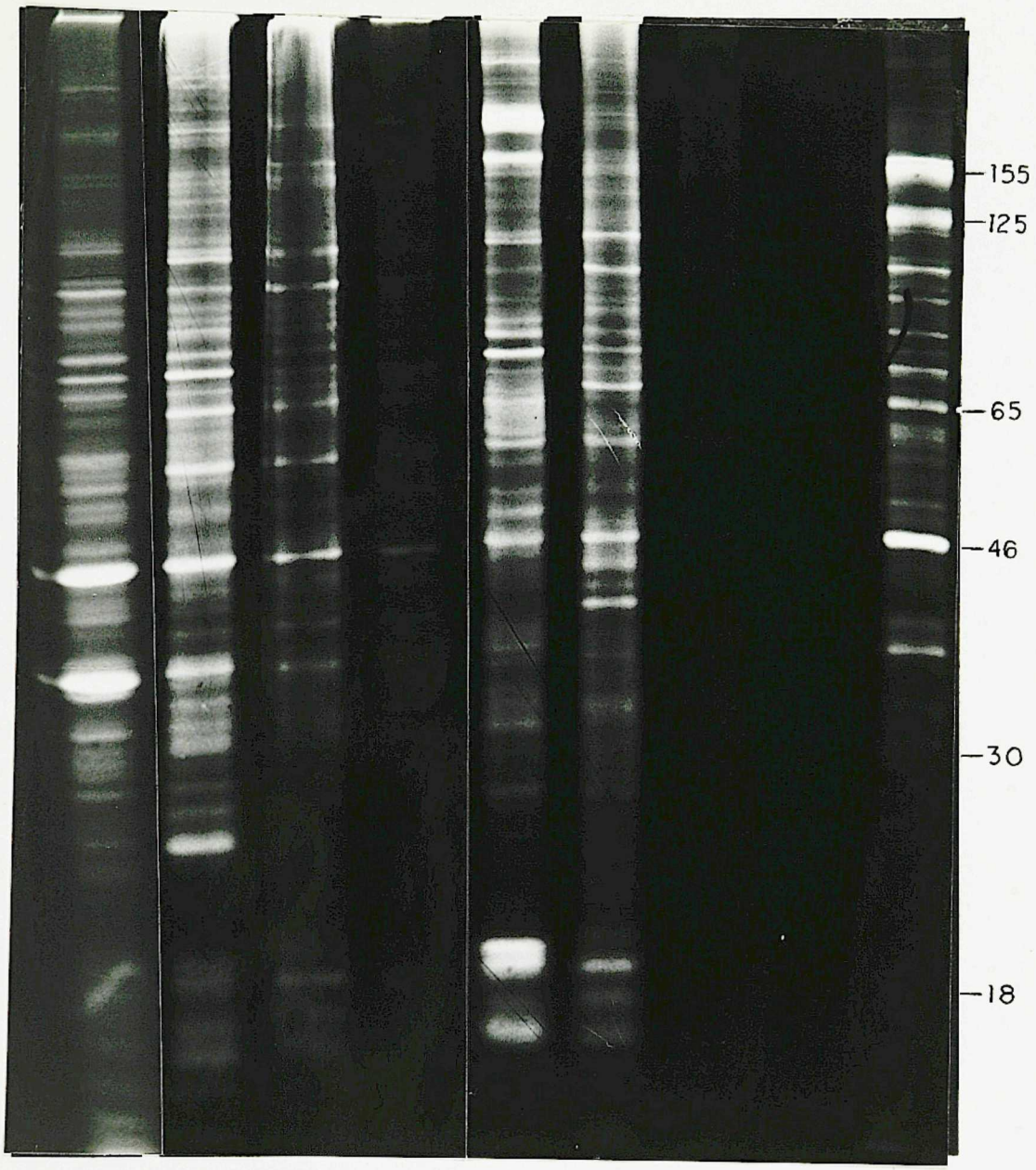

155
125
65
46
30
18
.2M .6M 1M 2M .2M .6M 1M 2M VZ
SINGLE STRANDED
DOUBLE STRANDED

Figure 37. Autoradiogram of the VZV infected cell (HFF) DNA binding proteins. Lanes 0.2M, 0.6M, 1M and 2M represent the acetone precipitated proteins from single stranded (left) and double stranded (right) DNA cellulose columns with increasing NaCl concentrations. Lane VZ is the infected cell extract used to obtain the DNA binding proteins. Molecular weights of the major DNA binding proteins of VZV infected cells are listed on the left.

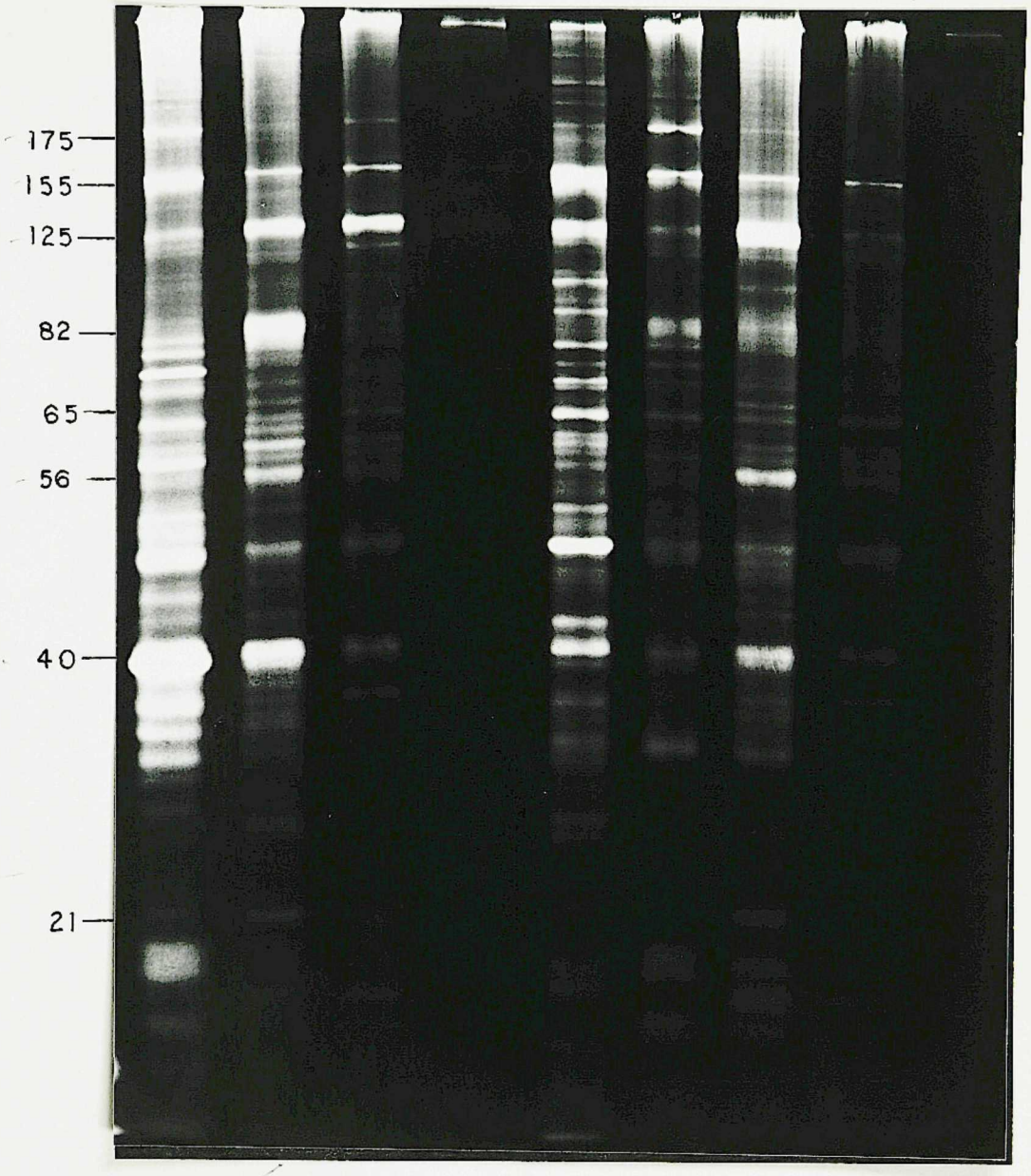
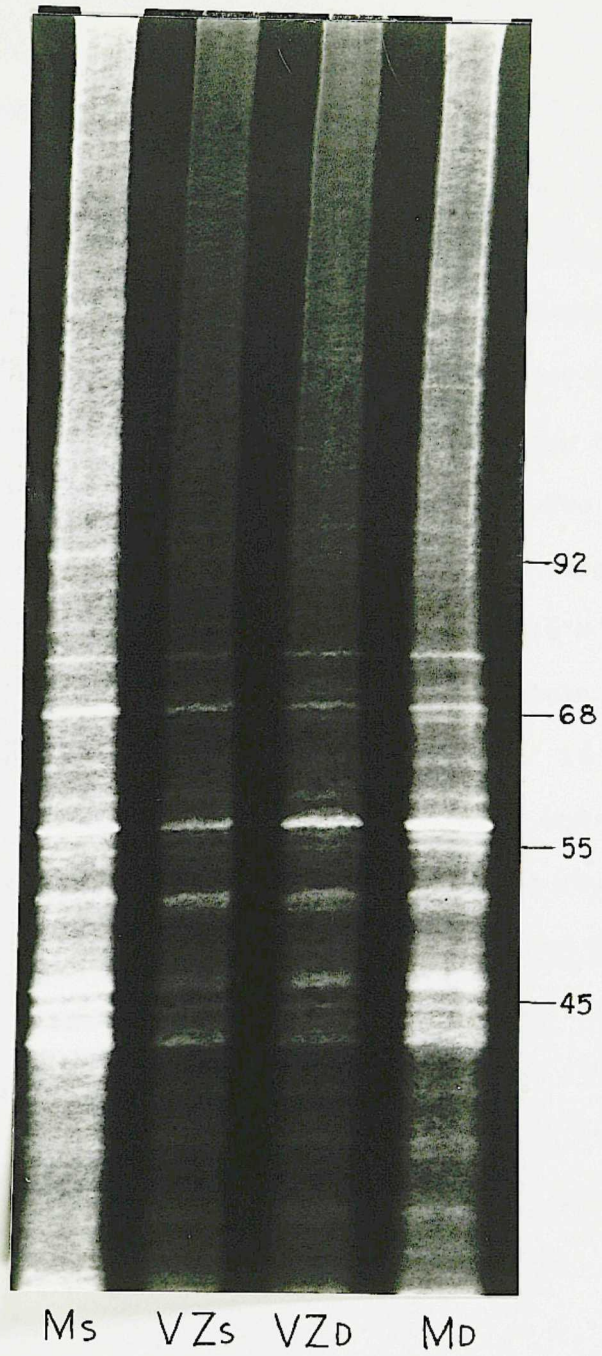

175
155
125
82
65
56
40
21
.2M .6M 1M 2M VZ .2M .6M 1M 2M
SINGLE STRANDED
DOUBLE STRANDED

Figure 38. Autoradiogram of proteins washed from single and double stranded DNA cellulose columns using 4.0 M guanidine hydrochloride. Lanes Ms and Md represent uninfected cell proteins from single stranded and double stranded DNA cellulose columns respectively. Lanes VZs and VZd represent VZV-infected cell proteins from single and double stranded DNA cellulose columns respectively.


92
68
55
45
Ms
VZs
VZD
MD

Peak fractions from the DNA cellulose column were pooled and the proteins were precipitated and subjected to SDS-PAGE analysis. Examination of the resulting autoradiograph shown in Figures 36 permitted identification of uninfected cell DNA binding proteins for comparison to infected cell DNA binding proteins. Figure 37 shows DNA binding proteins of VZV infected cells and allowed the identification of a minimum of seven VZV induced DNA binding proteins (175K, 155K, 125K, 82K, 56K, 40K and 21K). All of these polypeptides bind to single-stranded DNA although with varying affinities. Two major single-stranded DNA binding proteins are the 125K and 82K species. Approximately 50% of the 125K protein elutes with the 0.6 M wash and the remainder elutes with the 1.0M wash. The 125K protein also binds to double-stranded DNA cellulose columns from which it is completely eluted by a 0.6 M NaCl wash. The molecular weight and elution properties of this polypeptide are very similar to those reported for the major HSV-1 DNA binding protein ICP8 (128K, Powell et al, 1981). The 82K species bound to single-stranded DNA cellulose columns but not to double stranded DNA columns. The 82K protein single-stranded DNA interaction withstood a 0.2 M wash but was completely eluted with 0.6 M NaCl. The remainder of the polypeptides listed above bound to both single and double- stranded DNA with apparently equal affinity based on these relatively crude criteria. Most of these polypeptides were eluted with 0.6 M NaCl with some residual material eluting at 1.0 M.

Essentially all of the labeled proteins were eluted by this procedure. A 4.0 M guanidine hydrochloride wash which was designed to strip the columns of residual, tightly bound proteins did not result in elution of any significant additional labeled material (Figure 38).

The 155K polypeptide is, as discussed above, the major capsid protein and the protein blot analysis of VZV nucleocapsids confirms that the major capsid protein has an affinity for VZV DNA (see Figure 41) as well as other DNAs. The 175K protein is a structural component of the virion (as was seen in Figures 23 and 27) but it is not present in purified nucleocapsids. The protein therefore may be analogous to the tegument protein of HSV-1 and to the virion basic phosphoprotein found in human and simian CMV virions (Gibson et al., 1983). Protein blotting studies below also showed that this polypeptide binds to DNA (Figure 41). The 40K DNA binding protein is also a structural component of the virion as shown in Figure 21. The remaining ^{35}S-labeled VZV DNA binding proteins identified in this study (56K and 21K) appeared to be non-structural since they cannot be detected in the virion (Figure 21). The 21K protein has the same DNA binding characteristics as the 21K HSV-1/HSV-2 DNA binding protein.

It has been reported in HSV infected cells that some DNA binding proteins are phosphorylated and that phosphorylation alters their DNA binding properties (Wilcox et al., 1980). In order to determine if phosphorylation of VZV DNA binding proteins altered their DNA binding properties, VZV infected and mock infected cells were labeled with ^{32}P as described in Materials and Methods. Extracts for DNA cellulose chromatography were prepared, absorbed on either single or double-stranded DNA cellulose columns and eluted with the same salt steps as were the ^{35}S-labeled proteins. The elution profiles were essentially identical to those of the ^{35}S-labeled proteins. The resulting autoradiograms (Figures 39 and 40) indicated that 10-12 phosphorylated polypeptides bind to DNA. In contrast, very few uninfected cell DNA

Figure 39. Autoradiogram of ^{32}P labeled single stranded DNA binding proteins. The left half of the autoradiogram represents the uninfected cell phosphoproteins and the right half represents VZV-infected cell phosphoproteins. Lane M is the uninfected cell extract loaded onto the column and Lane VZ is the infected cell extract loaded onto the column. Lanes .2, .6. 1, and 2 are the acetone precipitated phosphoproteins of peak fractions from column eluates with increasing NaCl concentration.

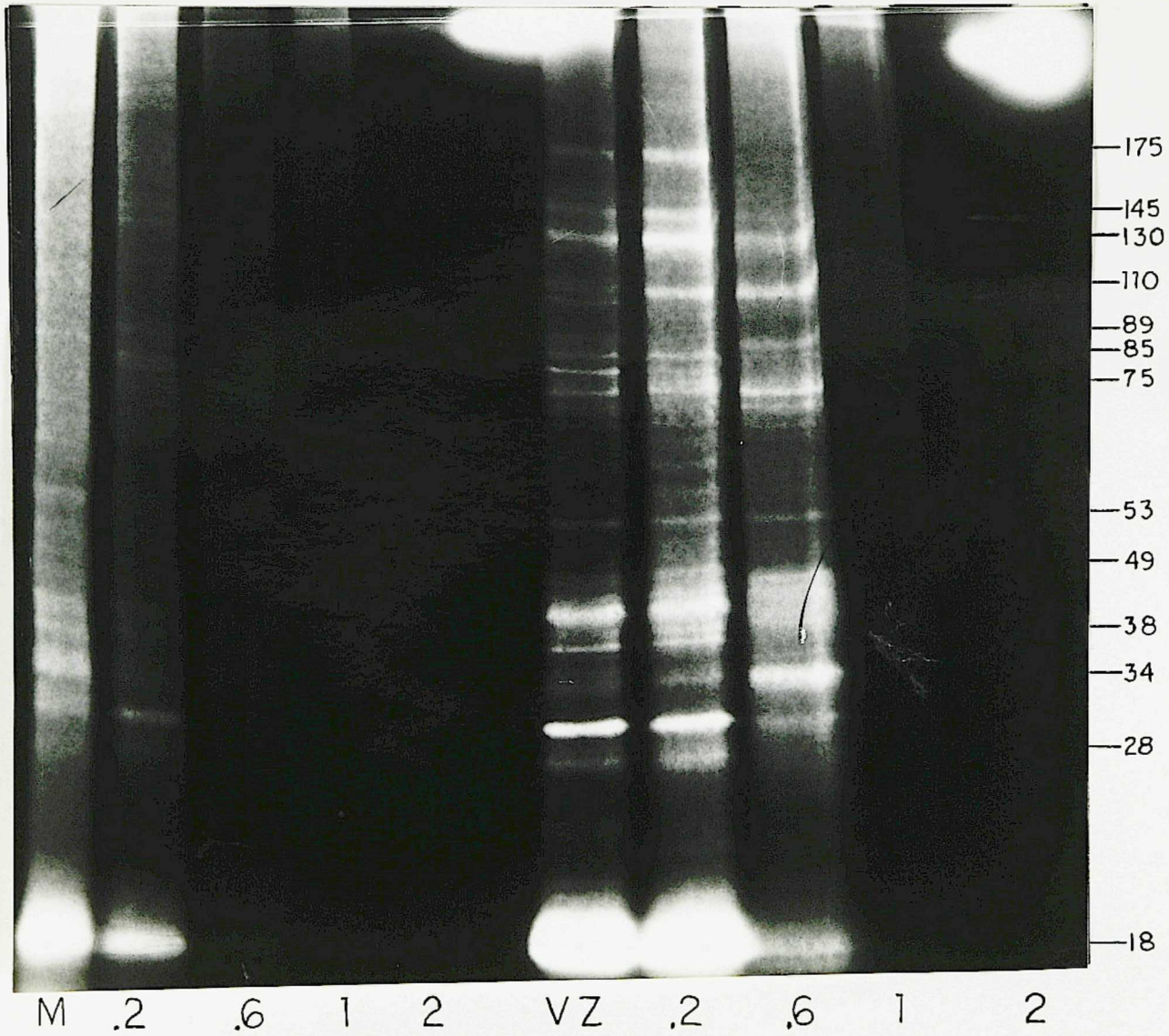
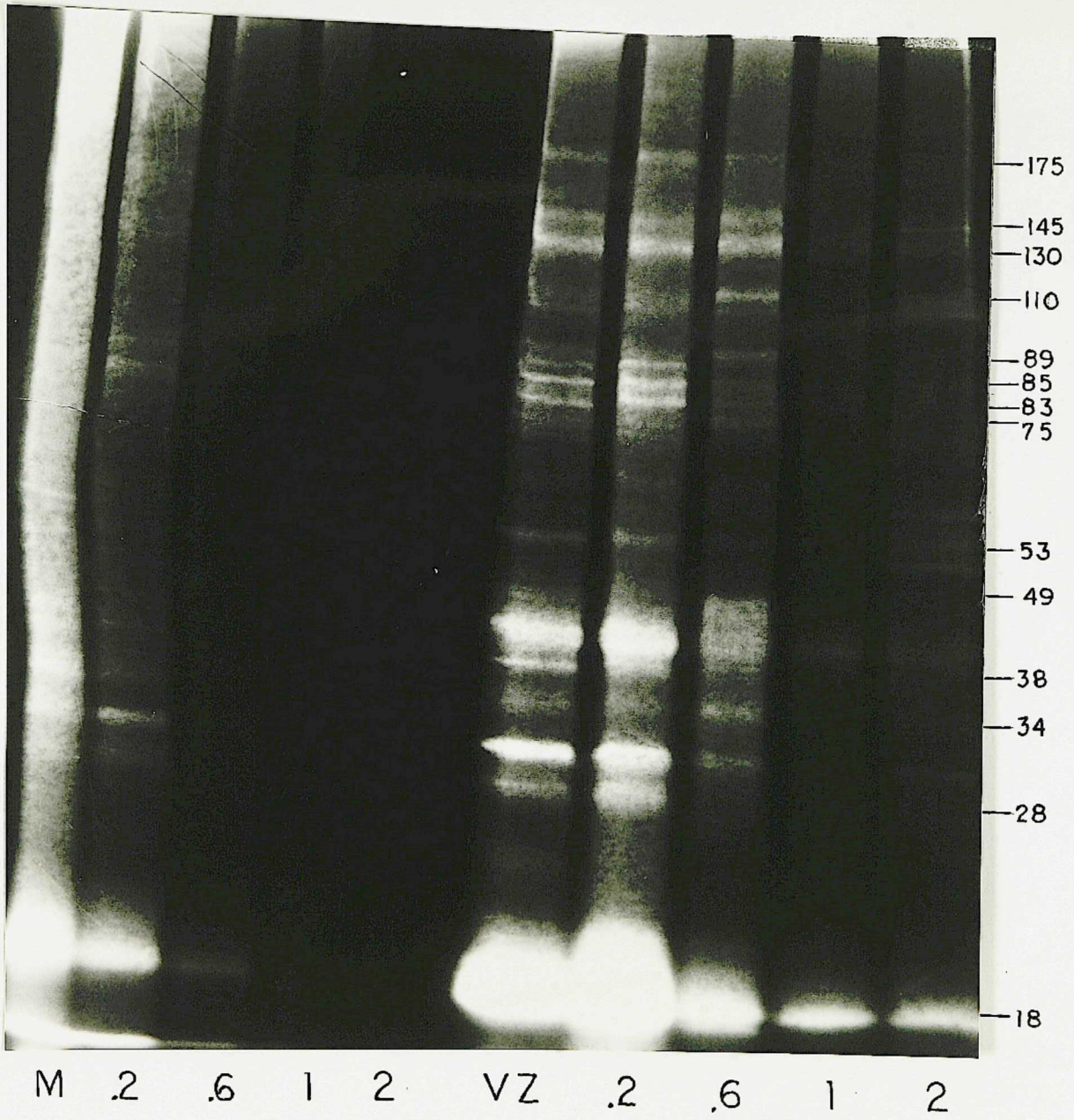

175
145
130
110
89
85
75
53
49
38
34
28
18
M .2 .6 1 2 VZ .2 .6 1 2

Figure 40. Autoradiogram of ^{32}P labeled double stranded DNA binding proteins. The left half of the autoradiogram represents the uninfected cell phosphoproteins and the right half represents the VZV-infected cell phosphoproteins. Lane M is the uninfected cell extract loaded onto the DNA cellulose column and lane VZ is the infected cell extract loaded onto the DNA cellulose column. Lanes .2, .6, 1, and 2 are the acetone precipitated phosphoproteins of peak fractions from column eluates with increasing NaCl concentrations.


175
145
130
110
89
85
83
75
53
49
38
34
28
18
M .2 .6 1 2 VZ .2 .6 1 2

binding proteins were phosphorylated. The ^{32}P-labeled DNA binding proteins ranged in molecular weight from 175K to 28K and are not necessarily identical to ^{35}S-labeled polypeptides of the same molecular weight. As was the case with ^{35}S labeled proteins, most of the DNA binding phosphoproteins bound to both single and double stranded DNA with no apparent differences in binding affinity.

Despite some similarities, only one of the major ^{35}S labeled DNA binding proteins could be unambiguously identified with the ^{32}P-labeled DNA binding phosphoproteins. This appears to be the putative 175K "tegument" protein present in ^{32}P labeled extracts and ^{32}P-labeled virions. The majority of the protein eluted from double-stranded DNA cellulose at 0.2M NaCl with some residual protein eluting at 0.6 M NaCl. Essentially all of the protein was eluted from single-stranded DNA cellulose with the 0.2 M NaCl wash. This behavior is similar to that found with the ^{35}S labeled proteins, although residual protein elution at 1.0 M was not observed.

The 145 K phosphoprotein is a new species which was not observed in ^{35}S labeled extracts. Either this polypeptide contains no methionine or, more likely, it is a minor species with relatively higher levels of phosphorylation. The protein appears to bind more strongly to double-stranded DNA than to single-stranded columns from which the majority of this protein elutes with the 0.2 M NaCl wash. This protein also appears to be a minor species which was detected only because of its phosphorylation.

The 130K DNA binding phosphoprotein is one of the major species and shares some properties with the major ^{35}S labeled DNA binding protein (128K). This phosphoprotein also binds to both single and double-strand-

ed DNA cellulose. The apparent affinities for single and double-stranded DNA cellulose, however, are reversed. The 130K phosphoprotein appears to bind more tightly to double than to single-stranded DNA. In both cases the protein is completely eluted from double-stranded DNA with 0.6 M NaCl. We suspect that the 130K protein is a phosphorylated derivative of the major VZV DNA binding protein (128K). Therefore, phosphorylation of the 128K protein may result in a decreased affinity of the protein for DNA. A similar result has been found for HSV-1 DNA binding proteins by Wilcox et al.,(1980).

Two other DNA binding phosphoproteins are worthy of note. One is a 110K polypeptide, the other is a 34 K polypeptide. Neither of these proteins is a major species in ^{32}P labeled cell extracts. Both bind better to single rather than to double-stranded DNA with the 34K species appearing to bind almost exclusively to single-stranded DNA.

The lowest molecular weight DNA binding phosphoproteins in VZV-infected cells are a 29K-30K species and a 27K-28K species which are also major uninfected cell phosphoproteins. These bind to both single and double-stranded DNA cellulose columns and are more intensely labeled in infected cells. The likelihood is that these are host cell-coded phosphoproteins, possibly subject to phosphorylation by a viral kinase.

In addition to these proteins, there are two diffuse series of bands between 89-75K and 49-38K which appear in ^{32}P labeled infected cell extracts. A majority of these proteins bind to both single and double-stranded DNA cellulose columns. Roughly equivalent amounts are eluted by 0.2 M and 0.6 M NaCl washes. Some residual material is eluted at 1.0 M and 2.0 M NaCl. The amount of protein eluted with the 2.0 M NaCl from the single and double-stranded columns is surprising and indicates that

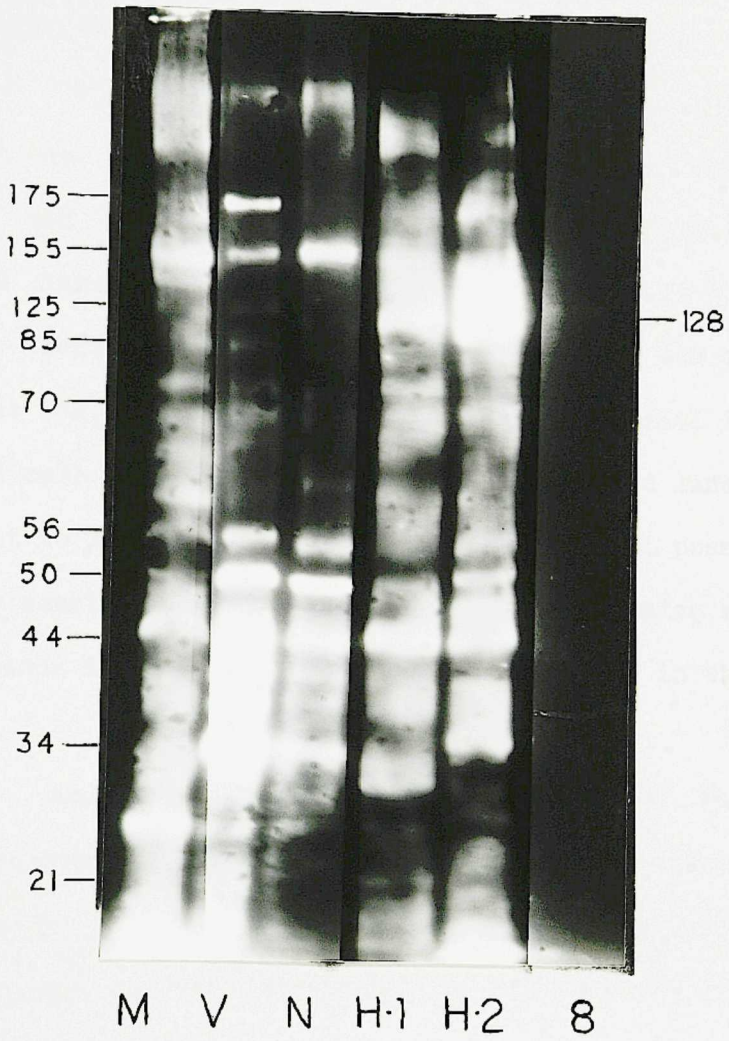
some phosphoproteins remain tightly bound to the DNA. The intense bands at 18K are probably histones. It is interesting that they are always more marked in the infected cell extracts and eluates.

Blotting of VZV polypeptides to DNA

In order to further define VZV DNA binding proteins and to gain information on their binding specificity, a series of VZV protein blots was prepared as described in Materials and Methods. These blots contained uninfected host cell proteins, VZV infected cell proteins, HSV infected cell proteins, and proteins from purified nucleocapsids. The blots were probed with a variety of radioactively labeled DNAs which included both homologous (VZV DNA) and nonhomologous (adenovirus and brominated poly dGdC [Z DNA]) DNAs. In doing this experiment were wished to see if any of the DNA binding proteins had a specific affinity either for VZV DNA or for Z DNA as opposed to B DNA. Z DNA is a configuration in which the DNA structure is more open and has been proposed as a site for the initiation of transcription or replication (Jovin et al., 1982). Figure 41 shows the results of probing a blot containing proteins from the above sources with ^{32}P- labeled double-stranded VZV DNA. The uninfected cell lane (M), as expected from the results of Figure 36, shows a large number of DNA binding proteins over the entire range of molecular weights. In contrast, both the VZV infected cell (V) and nucleocapsid (N) lanes show respectively 10 and 7 major bands corresponding to DNA protein interactions, ranging in the molecular weight from 175K to 21K.

Specifically the 175K, 155K, and 125K species identified previously and presumed to be the "tegument", major capsid, and major DNA binding

Figure 41. Autoradiogram of ^{32}P-labelled VZV DNA bound to the polypeptides of VZV-infected cells (V), VZ nucleocapsids (N), HSV-1 infected cells (H-1), HSV-2 infected cells (H-2), HSV-1 ICP 8 (8), and uninfected cells (M).


175
155
125
85
70
56
50
44
34
21
128
M
V
N
H-1
H-2
8

protein respectively, all of which interact with both single and double stranded DNA are present in the VZV infected cell lane. These results are completely in keeping with the locations and properties of these proteins as described in previous sections although the 125K band is not as intense as one would expect. There is a faint 68K band in the nucleocapsid lane which does not correspond to any of the bands seen in the VZV infected cell lane, perhaps because the protein is concentrated in the nucleocapsid. Two strong bands with molecular weights of 56K and 50K are seen in both the VZV infected cell and nucleocapsid lanes. There is also a group of at least five, and possibly more, bands ranging in molecular weight from 44K to 30K with a major band at 34K. Some lower molecular weight material is seen in the VZV infected cell lane as opposed to the nucleocapsid lane, but the gel is difficult to interpret in this region. It is not possible to draw any further conclusions, however, since there is also a substantial number of bands in this molecular weight range seen in the uninfected cell lane.

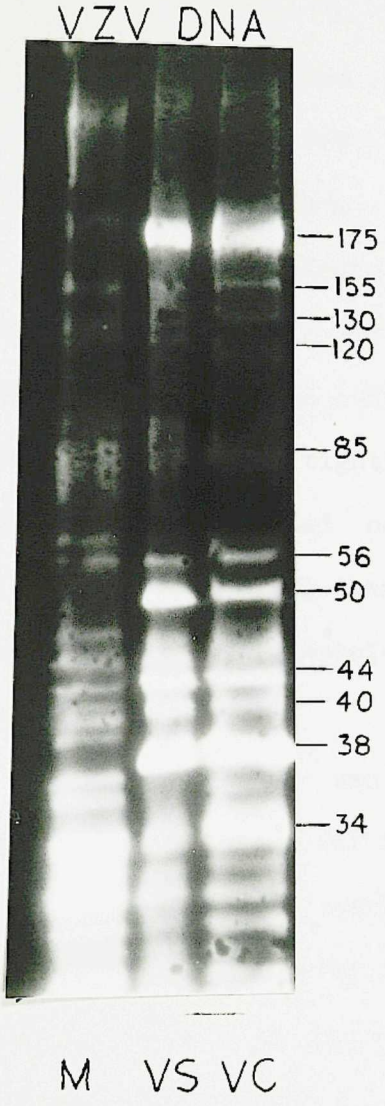
The HSV-1 and HSV-2 infected cell lanes have many bands, few of which are well defined. This makes detection of virus-specific DNA binding proteins difficult. There are, however, similarities to previously published reports including a very intense area at about 128K (Bayliss et al.,1975). A HSV-1 ICP 8 polypeptide prepared by the method of Powell et al., (1981), and used as a marker, is known to have a molecular weight of 128K and does show a faint band in this region (lane 8).

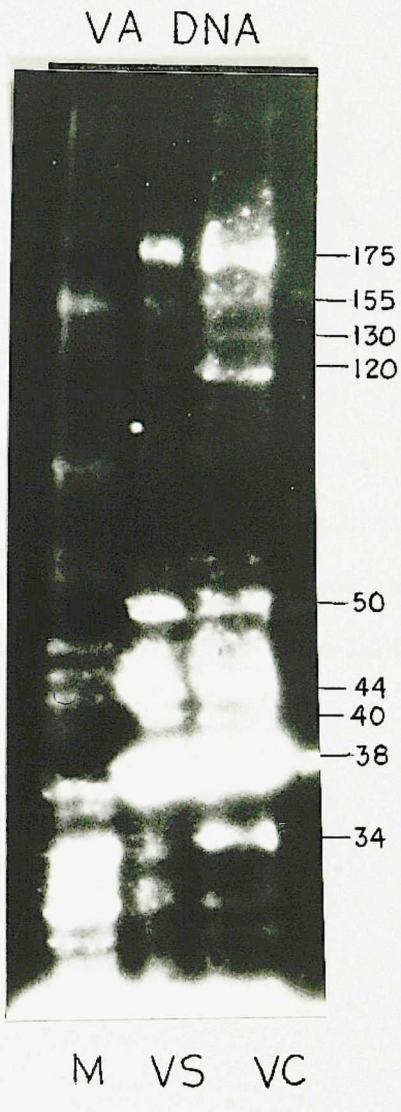
One of the methods for assessing relative strengths of binding of protein to DNA is to bind at low salt and wash with increasing salt

concentrations. Normally, if protein binds strongly to DNA it will resist elution until high salt concentrations; this has successfully been used with DNA binding columns (e.g. Figures 36 and 37). It would be useful if this DNA blotting procedure could also be used to assess DNA binding strengths. A blot hybridized to DNA at low salt concentrations was washed with buffers of increasing salt concentrations and between washes the blot was set up for autoradiography. It was clear from these data (not shown) that DNA eluted more readily from certain proteins; such proteins might be considered to be relatively weakly DNA binding. An example is the 175K phosphoprotein which we have already shown eluted from a DNA cellulose binding column at a low salt concentration. Thus the blotting procedure can also be used to estimate relative binding strengths of proteins.

It was important to ascertain if any of the blotted VZV proteins preferentially bound to VZV as opposed to heterologous DNA. The heterologous DNA used was the VA gene of adenovirus cloned into the plasmid vector pBR 322, chosen because it was a gene from a different DNA animal virus that does not share any nucleotide sequences with VZV and was readily available. The VA gene of adenovirus encodes a small RNA similar to the 5S RNA of mammalian cells and the function of the VA RNA is not well understood (McGowan et al., 1982). Both the VZV and the cloned adenovirus DNAs were nick translated and incubated with a protein blot made up of uninfected cell proteins (lane M) and proteins from cells infected with VZV strain Scott (lane VS) and VZV strain Champ (lane VC). The blot was hybridized with the cloned VA gene first and set up for autoradiography. The blot was then washed in binding buffer with increasing NaCl starting at 0.2 M and ending at 2.0 M. Most

Figure 42. Protein blot analysis of the binding of VZV DNA versus the cloned VA gene of adenovirus. The polypeptides of uninfected cells (M) and cells infected with VZV strain Scott (VS) and VZV strain Champ (VC) were separated and transferred to nitrocellulose as described in Materials and Methods. The filter was hybridized with the cloned VA gene DNA and set up for autoradiography. The blot was then washed in binding buffer with increasing NaCl concentrations and reprobed with VZV DNA. Probes were ^{32}P-labelled by nick translation.

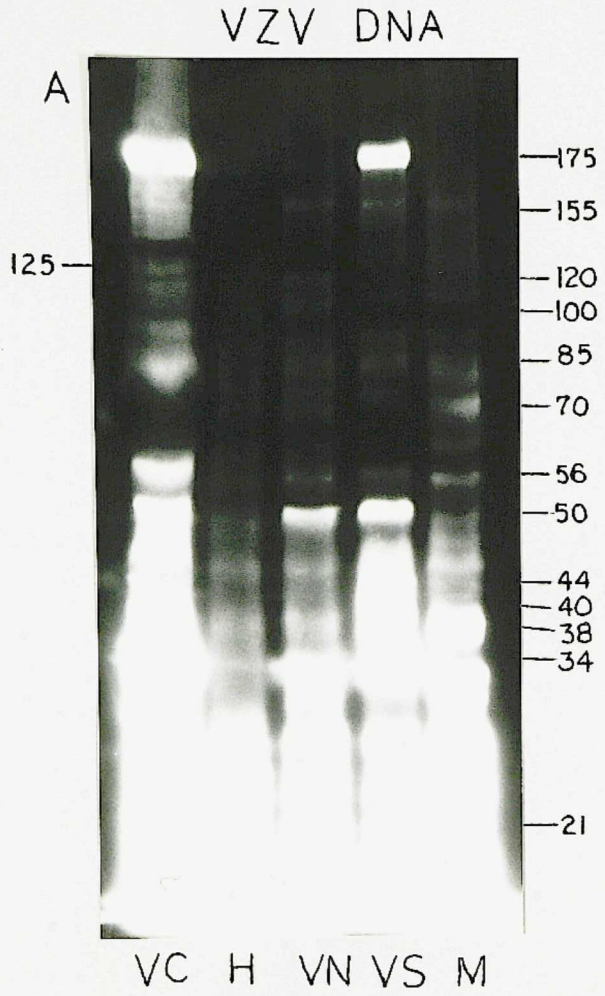

VZV DNA
175
155
130
120
85
56
50
44
40
38
34
M VS VC


VA DNA
175
155
130
120
50
44
40
38
34
M VS VC

of the DNA was removed as reexposure to X-ray film at this time showed no radioactivity present. The blot was then rehybridized with VZV DNA. Figure 42 shows the results obtained; the right panel shows the blot probed with the cloned adenovirus gene and the left panel the blot probed with the VZV DNA. Within the limits of the assay some general differences were evident. The majority of these differences appear to be a decreased affinity of VZV proteins for the adenovirus/plasmid sequences relative to VZV sequences. The major exception is the 38K protein, which in the infected cell case at least, appears to bind the exogenous DNA's more tightly than VZV DNA. Using the 175K protein as a standard, VZV infected cell proteins which appeared to have a higher affinity for VZV DNA than for adenovirus/plasmid sequences include a 85K species, a 56K species, and a series of lower molecular weight bands (36-25K).

Further blotting was attempted in order to determine if any proteins present in VZV infected cells and/or nucleocapsids showed affinity for the "Z" configuration of DNA. A protein blot containing VZV infected cell proteins, VZV nucleocapsid proteins, HSV-2 nucleocapsid proteins, and uninfected cell proteins was prepared. Figure 43 shows the results obtained with panel A showing the protein blot probed with VZV DNA and panel B the blot probed with the "Z" DNA (5' end-labeled poly dGdC). The blot was probed first with VZV DNA and then treated as mentioned above before reprobing with the "Z" DNA. Four infected cell proteins showed clear affinity for the "Z" DNA. These were the 175K "tegument" protein, the 56K, the 50K, and the 34K which had been identified as DNA binding in previous blots. As expected, the 175K protein band is absent in the nucleocapsid lane. No specific bands

Figure 43. Protein blot analysis of the binding of double stranded VZV DNA (A) versus "Z" DNA (B). Lane VC, VZV strain Champ infected cell polypeptides; lane H, HSV-2 nucleocapsids; lane VN, nucleocapsids of VZ strain Oka; lane VS, VZV strain Scott infected cell polypeptides; lane M, uninfected cell polypeptides. The proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose filter as described in Materials and Methods. The filter was first hybridized with ^{32}P-labeled VZV DNA and set up for autoradiography. The blot was then washed with increasing concentrations of NaCl in binding buffer and reprobed with ^{32}P-labelled "Z" DNA. Both blots were exposed to XAR-5 film for 8 hours.


VZV DNA
A
175
155
125
120
100
85
70
56
50
44
40
38
34
21
VC H VN VS M

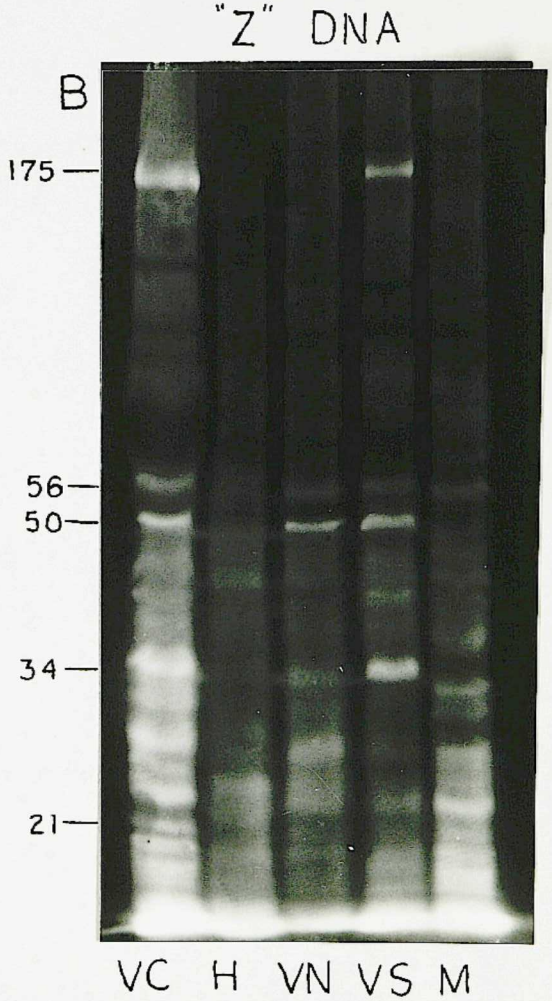

"Z" DNA
B
175
56
50
34
21
VC H VN VS M

Table 5. Summary of VZV DNA Binding Proteins

Single-stranded				Double-stranded				Protein Blot	
^{35}S		^{32}P		^{35}S		^{32}P			
M.W.	NaCl	M.W.	NaCl	M.W.	NaCl	M.W.	NaCl	VZV DNA	Z DNA
175	.2	175	.2	175	.2	175	.2	175	175
155	.2-1	145	.2	155	.2-1	145	.2-.6	155	
125	.2-.6	130	.6	125	.6	130	.6	125	
		110	.2-.6			110	.6		
82	.6	89 | 75	.2-.6			89 | 75	.2-.6	85	
56	.2	53	.2-.6	56	.6	53	.2-.6	56	56
40	.6	49 | 38	.2-.6	40	.6	49 | 38	.2-.6	50	50
		34	.6			34	.6	34	34
21	.6			21	.6				

Note: The DNA binding proteins were identified either by DNA cellulose chromatography (Figures 36, 37, 39, and 40) or by the protein blot procedure (Figures 41, 42, and 43). The abbreviations are: M.W., apparent molecular weight in thousands; NaCl, molar salt concentration at which the majority of the protein is eluted; [35]-S, [35]-S-methionine labeled proteins; [32]-P, [32]-P-orthophosphate labeled proteins.

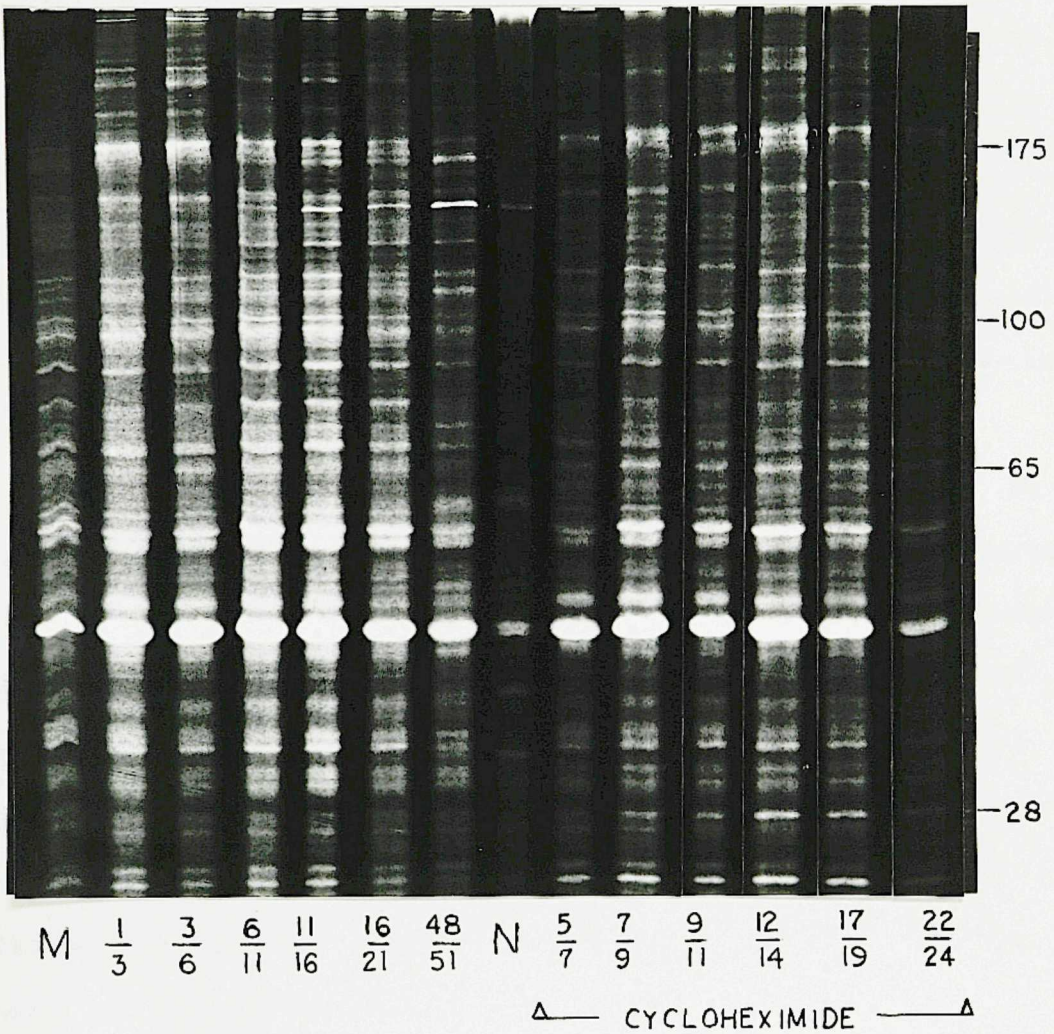
indicating "Z" DNA affinity were seen, either in the uninfected cell lane or the HSV-2 nucleocapsid lane under the conditions used in these experiments. A summary of VZV DNA binding proteins is presented in Table 5.

"Immediate Early" VZV polypeptides

One of the most interesting populations of proteins in herpesvirus infected cells is the "immediate early" subset. These are made in the absence of viral protein synthesis and initiate the replicative cycle. Their mRNAs accumulate in the presence of cycloheximide and when the cycloheximide is removed, the proteins are immediately made. They may also have a role in controlling host functions and switching viral synthesis to "early" and "late" phases.

One approach to the characterization of such polypeptides in other herpesvirus infected systems has been through the use of cycloheximide block and release of virus infected cells. With this technique it was hoped that the detection of "immediate early" VZV proteins would be possible. The technique involves incubation of infected cells with cycloheximide for a time immediately following infection, and then releasing the block by washing the cycloheximide from the infected cells. Label can be added at that point. The result of such an experiment is shown in Figure 44. The left half represents a PAGE of the polypeptides produced over a 51 hour time course without cycloheximide that were run simultaneously with polypeptides from infected cells treated with cycloheximide for 5 hours before being released from the block. The blocked and released cells are on the right side of the gel. Three VZV polypeptides appear to arise rapidly after release from the cycloheximide block. The most conspicuous is at 180-175K with the

Figure 44. Autoradiogram demonstrating the effect of cycloheximide block and release on the synthesis of VZV polypeptides. The left half represents the polypeptides of VZV produced over a 51 hour time course without cycloheximide. Uninfected cells are in lane M and VZV nucleocapsid proteins are in lane N. The right half represents the polypeptides produced after newly inoculated cells were blocked with 50 ug/ml of cycloheximide for 5 hours and then released by changing media. Numbers indicate the beginning (top) and the end (bottom) of labeling period (in hours) with ^{35}S-methionine. Numbers on the left represent hours post inoculation and numbers on the right represent hours post cycloheximide block and release. Three proteins are apparent after cycloheximide release (175K, 65K, and 28K) suggesting that they are "immediate early" polypeptides of VZV.


175
100
65
28
M
1/3
3/6
6/11
11/16
16/21
48/51
N
5/7
7/9
9/11
12/14
17/19
22/24
CYCLOHEXIMIDE

others at 65K and 28K. There is also some minor activity apparent at 100K. The rapid appearance of these proteins after release from cycloheximide suggests that they are "immediate early" polypeptides of VZV.

Cell free virus inoculum

In searching for "immediate early" polypeptides, we were concerned that the use of whole infected cells as a source of virus might make interpretation of the experimental data difficult. The reason for this might be the lack of synchrony in the infection and the possibility that the cells used as inoculum might produce proteins which could be falsely identified as "immediate early". Therefore, the experiment was repeated with an inoculum that was sonicated to insure that no intact cells were present.

The major difficulty in using sonically disrupted cells is the tremendous decrease in infectivity. This phenomen has been reported by many others (e.g., Taylor-Robinson and Caunt, 1972) and is certainly the case with the HFF cell line used in these studies. It is not clear why cell free extracts have such low infectivity, but it has been proposed that the virus has a fragile envelope which may be damaged by the sonication treatment (Cook and Stevens, 1968). Figure 45 shows the effects of ultrasonic treatment of the inoculum prior to infection. Two sets of cells were infected with a higher than normal "MOI" of one infected cell to three uninfected cells. One set received a "sonicated" inoculum. Such a "sonicated" virus preparation has a titer of no more than 10^4 pfu/ml on HFF cells. In the "normal" set 10% CPE was apparent by 20 hours post infection whereas CPE was not apparent in the set which received the "sonicated" inoculum until 56 hours post infection. Using the appearance of the major capsid protein as a marker, this

Figure 45. Autoradiogram comparing the effects of sonicating the inoculum (right half) to inoculating cultures with infected cells (left half). Cells were infected with a higher than normal "MOI" of one infected cell to three uninfected cells. Lane M represents uninfected cells. Numbered lanes represent the beginning (top) and the end (bottom) of the labelling period (in hours) with ^{35}S-methionine.

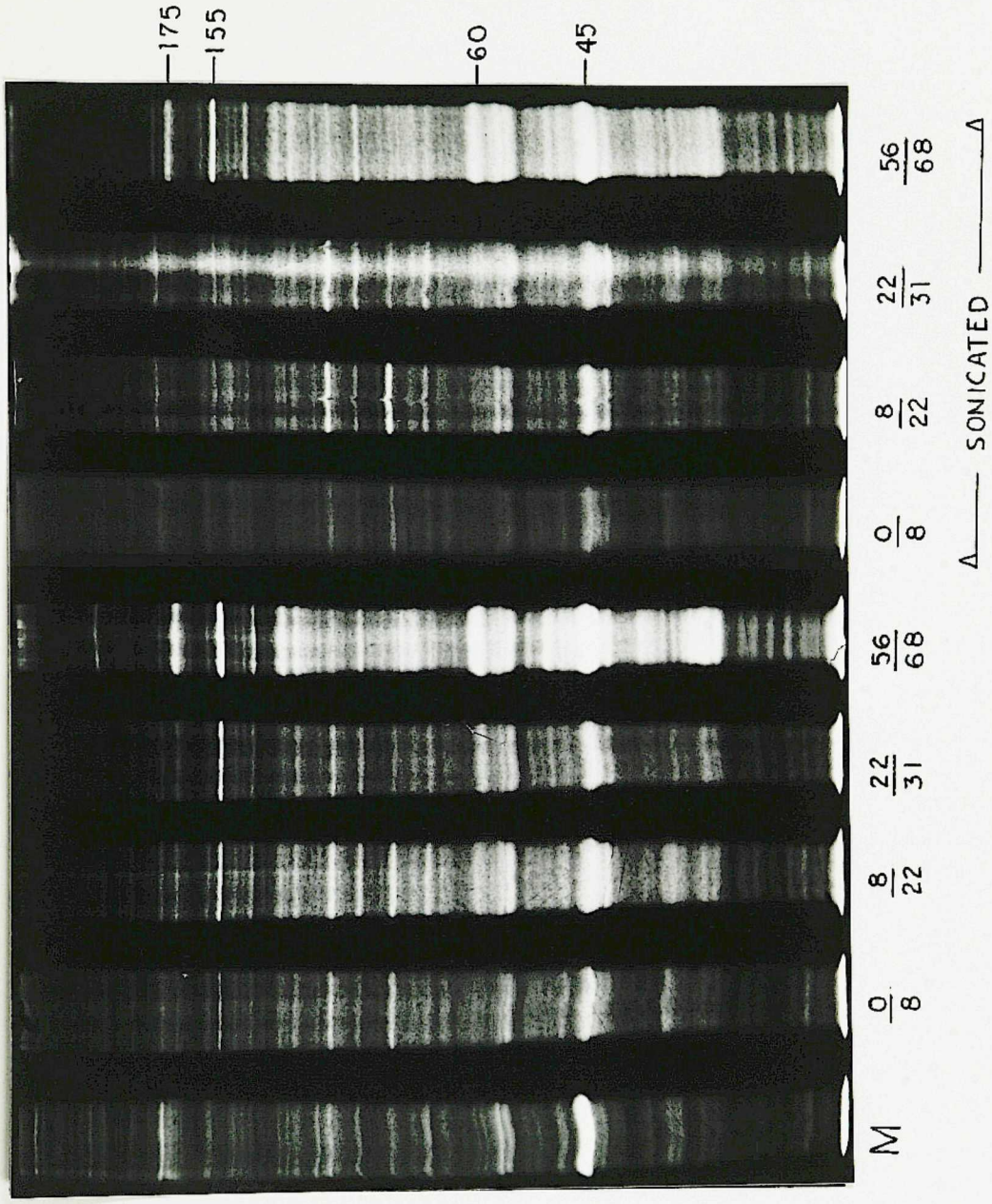
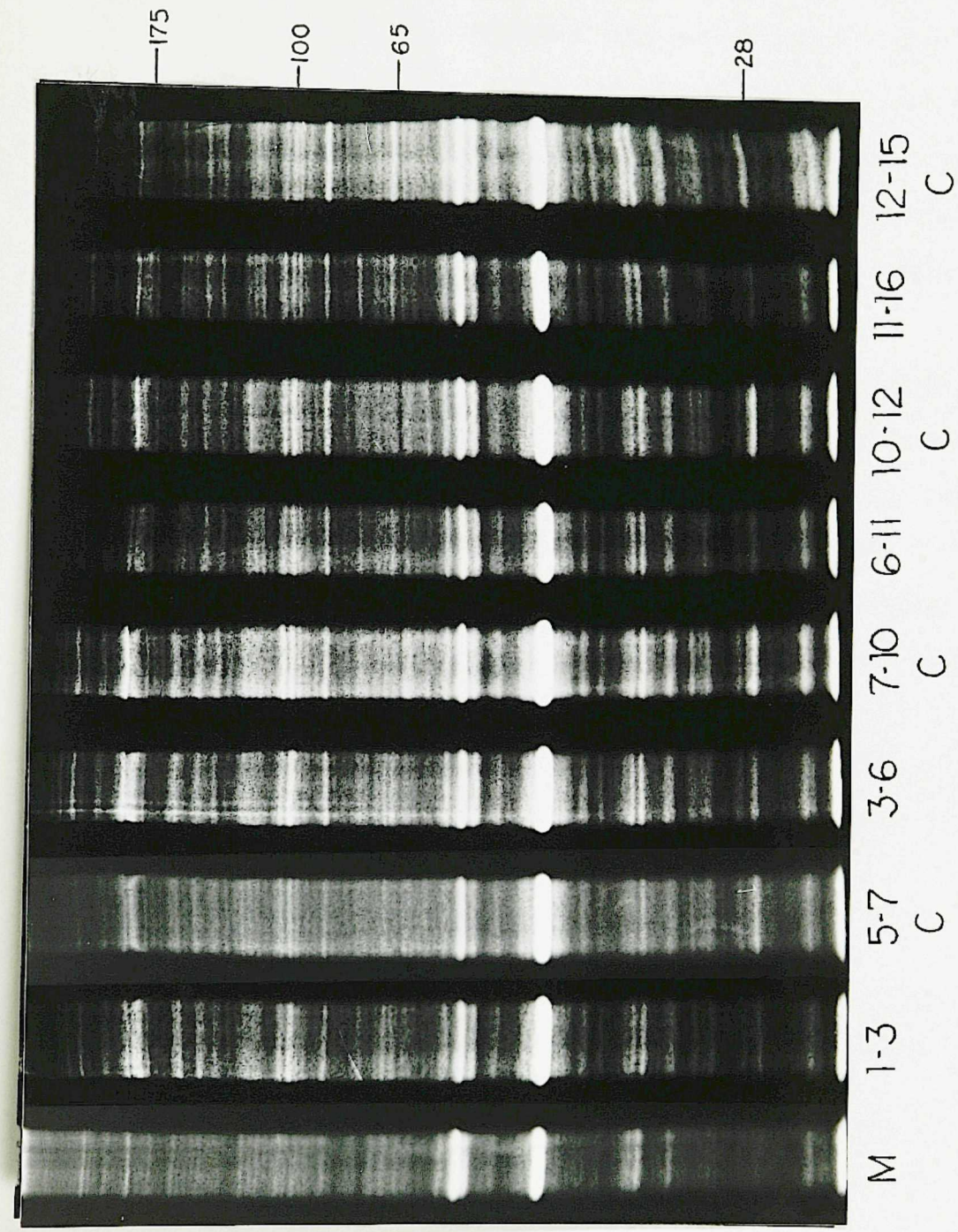

175
155
60
45
M
0/8
8/22
22/31
56/68
0/8
8/22
22/31
56/68
SONICATED

Figure 46. Autoradiogram demonstrating the effects of cycloheximide on the synthesis of polypeptides in VZV infected cells. Experimental conditions were the same as in Figure 44 except that the inoculum was sonicated prior to infection. Lane M represents uninfected cells. Numbered lanes indicate the beginning and end of the labelling period (in hours after inoculation). Those with a C are cycloheximide blocked and released and the numbers indicate the hours after release. The three proteins (175K, 65K, and 28K) seen in Figure 44 are also present following inoculation with sonicated virus.


175
100
65
28
M
1-3
5-7
C
3-6
7-10
C
6-11
10-12
C
11-16
12-15
C

decrease in infectivity can be seen in the protein patterns shown in Figure 45. In the cultures inoculated with infected cells (left side of gel) the major capsid protein can be seen by 8 hours post infection. In the cells grown in the "sonicated" inoculum (right side of gel) the major capsid protein is not readily apparent until 56 hours post infection. By this time CPE was only 25% whereas in the normal set CPE was 100% by this time.

However, the results from Figure 45 were encouraging enough to try a sonicated virus inoculum in a cycloheximide block and release experiment. Figure 46 shows that, even if the viral inoculum is sonically disrupted, proteins with the molecular weights of 180-175K, 100K, 65K, and 28K all arise after release from cycloheximide, confirming their "immediate early" nature. Conditions altered in other experiments included blocking the infected cells with cycloheximide for 7,10, 15 and 20 hours as well as for 5 hours before release and labeling. In all cases the same molecular weight species arose after release from cycloheximide (data not shown).

Immunological characteristics of VZV polypeptides

Zoster immune globulin (ZIG) is used in some instances to modify the severity of chickenpox infections (Weller,1983) and this pooled human IgG preparation presumably contains antibodies which are directed primarily against the major antigens of VZV, amongst others. It was therefore important to identify which proteins reacted with these antibodies against Varicella-Zoster virus. Accordingly, a "Western blot" analysis was performed using ZIG against VZV infected cells, virions, nucleocapsids, uninfected cells and HSV-1 infected cells. The results are shown in Figure 47. The major viral polypeptide reacting with ZIG

Figure 47. A Western blot analysis of the binding of Zoster immune globulin (ZIG) against VZV-infected cells (lanes W, strain Webster; S, strain Scott, and O, strain Oka), HSV-1 infected cells (lane H), VZ virion preparation (lane V), VZ nucleocapsid preparation (lane N) and uninfected cells (lane M). Cell extracts were fractionated on a 12% polyacrylamide gel and transfered to nitrocellulose sheets. The sheets were allowed to bind ZIG and then incubated with ^{125}I Staph A protein as detailed in Materials and Methods.

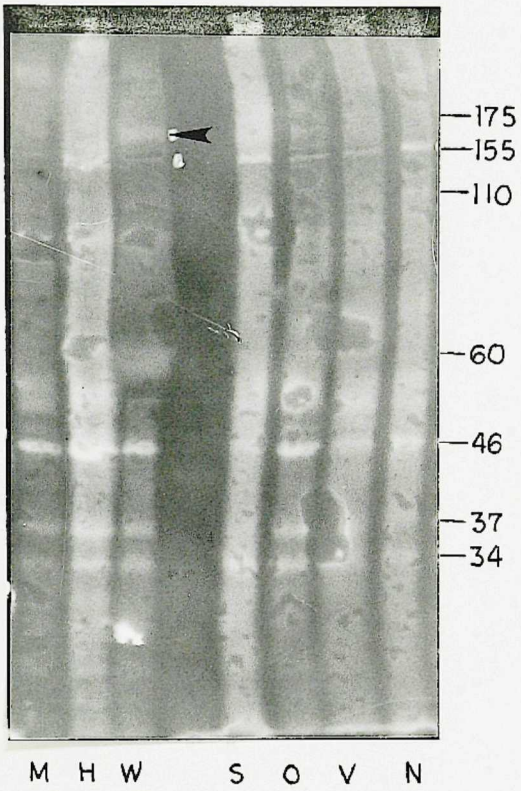
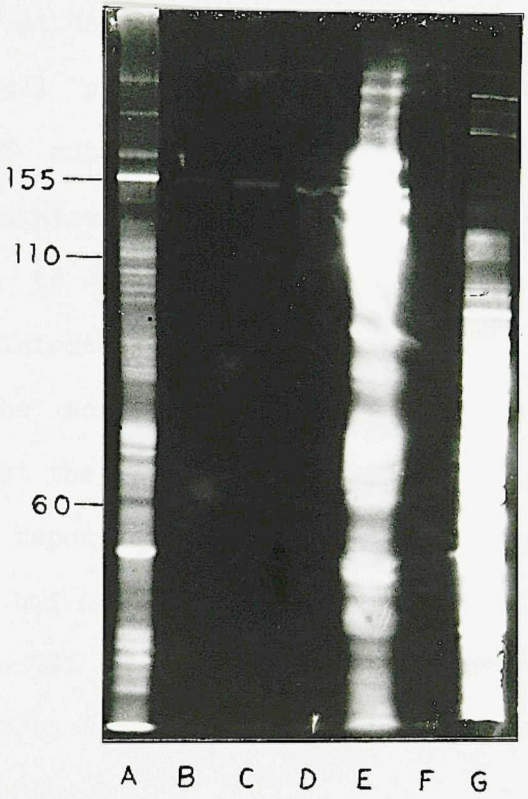

175
155
110
60
46
37
34
M H W S O V N

Figure 48. A Western blot analysis of the binding of hyper-immune rabbit anti-HSV-1 sera to proteins from VZV infected HFFs (lanes B,C,D), HSV-1 infected BHK cells (lane E), and uninfected HEL cells (lane F). Lanes A and G are ^{35}S-methionine labelled VZV and HSV-1 infected cells respectively and used as viral protein markers. The blot was performed during a collaborative study with Dr. A Buchan, The University of Birmingham, U.K.

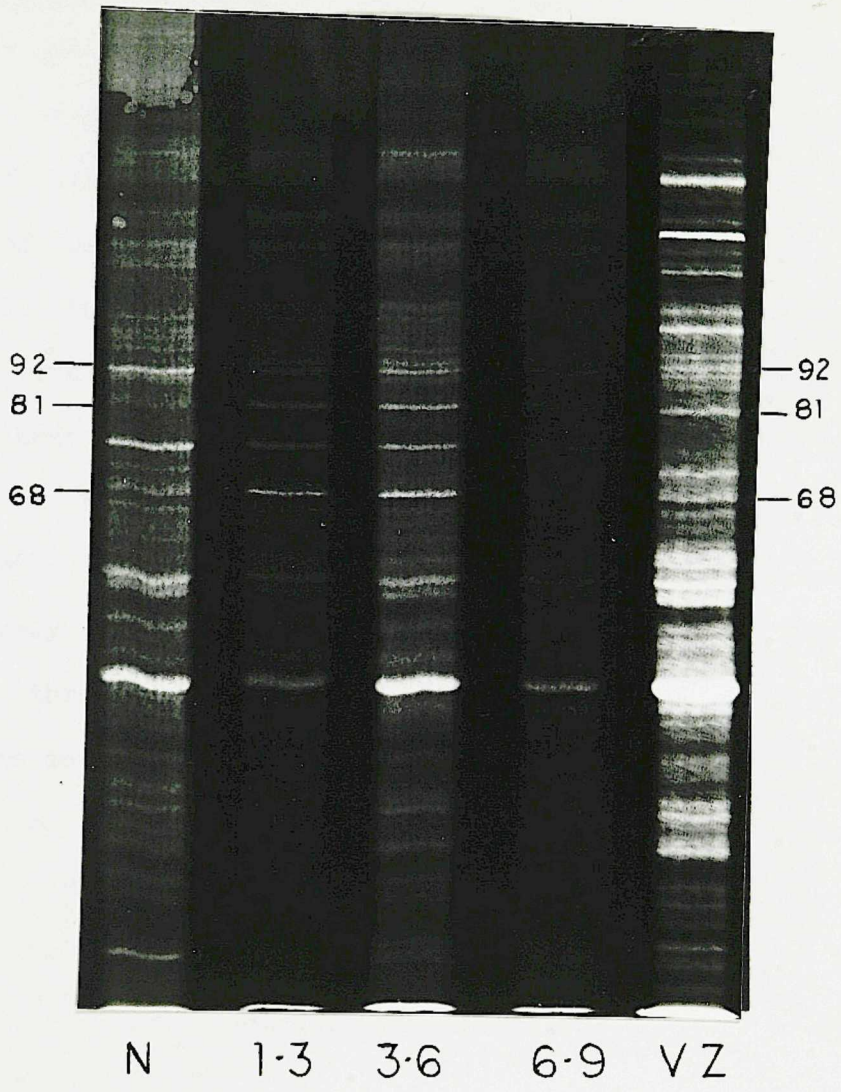

155
110
60
A B C D E F G

appears to be the 155K major capsid protein. In the Webster infected cell lane the 175K protein also appears to have bound ZIG (see arrow), but the virion preparation from VZV strain Oka did not (lane V). There is also some binding evident in the 60K region in lane W. Curiously, three bands at 46K, 37K, and 34K appear in all lanes including the uninfected cell preparation (M). Perhaps VZV infection induces the production of autoantibodies or there are cellular proteins antigenically recognized by pooled human IgG. Conceivably, the "Staph A" protein used to visualize binding may adhere to proteins in both infected and uninfected cells.

One of the more interesting observations was that the ZIG also reacted against the 155K major capsid protein of HSV-1 (lane H). Ross et al. (1965) reported that VZV and HSV share at least one antigen, but that result had never been analyzed in detail. Since the above result may be due to HSV antibodies in the ZIG preparation, a second experiment was run in which specific HSV-1 antibody (supplied by Dr. A. Buchan of the University of Birminghan, U.K.) was used as the probe. Using hyperimmune rabbit anti-HSV-1 antibody, a Western blot was performed against VZV and HSV-1 infected cells. The HSV-1 lane shows, as expected, heavy antibody binding; but in the VZV lane only three polypeptides were detected at 155K, 110K, and 60K (Figure 48). The 155K protein is presumably the major capsid protein of VZV. The report of Shiraki et al. (1982) indicated cross-reactivity with 64K and 55K VZV polypeptides. However, this is the first report of cross-reactivity with the 155K major capsid protein.

Future studies on the immunological relationships of VZV will be made easier now that monoclonal antibodies are beginning to become

Figure 49. Autoradiogram of the heat shock proteins of human foreskin fibroblasts. HFFs were stressed for one hour at 43°C, labelled with ^{35}S-methionine, and subjected to SDS-PAGE analysis as described in Materials and Methods. Lane N, non-stressed HFFs; lane VZ, cells 48 hours post VZV infection; numbered lanes, time in hours post shock cells were labelled. Three proteins at 92K, 81K, and 68K appear to be induced by heat shock.


92
81
68
92
81
68
N
1·3
3·6
6·9
V Z

available. Such experiments are now underway with murine and human monoclonal antibodies.

Heat shock/stress proteins

Recent publications (Nevins, 1982; Notarianni and Preston, 1982) indicated that certain proteins are induced in tissue culture as a result of trauma. These "heat-shock" proteins can be induced by a variety of conditions, ranging from increased temperatures to viral infections. In order to prevent misclassification of such stress proteins as viral-specific proteins in VZV infected cells, HFFs were subjected to heat shock and the resulting stress proteins were compared to proteins of VZV infected cells. Figure 49 shows the effect of heat shock in HFFs. After raising the temperature to 43° C for one hour and then quickly returning the cells to the normal 37° C incubation temperature, three proteins at 92K, 81K, and 68K appeared to be induced. These were detected by 1-3 hours post shock and their synthesis was declining by 6-9 hours. Comparison with VZV infected cells clearly shows in these a new 92K and an 81K protein and, possibly, a 68K species which also appear after infection. It therefore seems likely that these may not be viral, but may be cellular proteins produced in response to the infection

VZV thymidine kinase

One of the few VZV proteins which has been partially characterized, albiet rather poorly, is a thymidine kinase which has been reported in infected cells and which is presumably virus encoded (Cheng et al.,1979). Now that we have begun to define structural and functional polypeptides of VZV, it would be important to begin to define the VZV genome in terms of genetic and physical organization. To

date, no genetic mapping has been accomplished with VZV. A good candidate for the first gene to be mapped on the genome is the thymidine kinase gene since the enzyme represents a selectable marker, TK- cells are available, and the TK genes from other herpesviruses have been isolated and cloned. Using techniques that were successful with HSV, we initiated studies to transform mouse LTK- cells with VZV DNA and select TK+ transformants with HATG selection media. Cloned VZV EcoRI and BamHI fragments (Figure 51) as well as whole VZV DNA were used in the calcium phosphate precipitation transfection technique (Gram and Van der Eb, 1973). Although "control" transformations with both HSV-1 and HSV-2 cloned TK genes were routinely successful, TK could not be consistently induced with any VZV DNA fragments. Initial attempts with transfection of cloned VZV DNA fragments yielded one transformant with the EcoRI restriction fragments H,P,Q. The TK activity was similar to that of HSV-transformed cells in that it was sensitive to Ara-T (data not shown, Gentry and Aswell, 1975). In addition, the DNA from the initial H,P,Q DNA transformants was able to transform other TK- cells to TK+ phenotype. Unfortunately, repeated attempts at transfection with other H,P,Q DNA preparations were unsuccessful even though HSV controls were uniformly successful. Therefore, the H,P,Q transformant was probably a rare cellular revertant.

The lack of success after many attempts with this technique suggests to us that the VZV TK gene was cut in a critical spot (and hence inactivated) by either EcoRI or BamHI restriction enzymes. Using the whole VZV genome has also proved unsuccessful probably because the large amounts of DNA needed to give a proper gene dosage were simply not available. By analogy with other herpesviruses' TK genes, the VZ TK

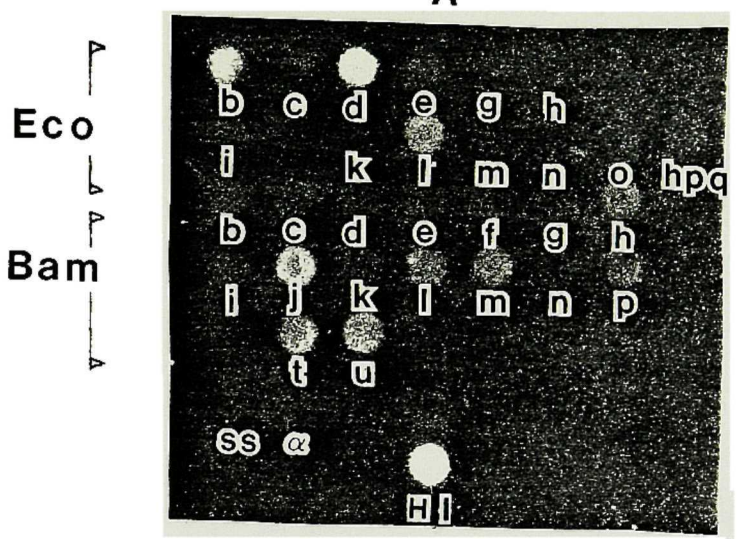
gene is likely to represent approximately 2.5% of the whole genome. Thus to transfect cells with 1 ug of TK gene DNA, we would need 40 ug of VZV DNA, an amount which would be very difficult to prepare.

As an alternative, hybridization experiments were performed using the specific HSV-1 and HSV-2 TK gene DNA as probes to locate a possible homologous region on the VZV genome. These probes also contained upstream promoter elements which may be homologous to regions of VZV DNA other than the TK promoter region, a possible problem in interpreting our data. Figure 50 shows the results of HSV-1 TK DNA hybridized to the various cloned fragments of VZV. Nonspecific interaction between the probe and the blotted DNA is always a potential problem in such hybridization experiments and must be taken into consideration. Nevertheless, after several hybridizations with the TK gene, either intact or excised from its plasmid carrier, a consistent pattern of hybridization emerges. This can be seen in Figure 51 which summarizes the dot blot hybridization data between HSV and VZV. Two areas of the VZV genome appear to be homologous with the TK genes of HSV. The major area of homology appears to be at the right end of the unique long region while another region appears at the left of the unique long region. A recent publication on the general homology of VZV and HSV-1 DNA indicates that the gene arrangement in the UL VZV may be inverted relative to the UL in HSV-1 (Davidson and Wilkie, 1983). Our data showing hybridization of the HSV TK DNA to the right end of the unique long region of VZV would support this hypothesis.

In addition, a region larger than that expected to encode for the VZV TK was detected by hybridization of our HSV-1 and HSV-2 probes. The reason for this may be that our probes contained promoter regions and

Figure 50. Dot blot hybridization of HSV-1 TK DNA to various clones of VZV DNA. In blot A the HSV-1 TK gene was removed from its plasmid carrier prior to probing whereas in blot B the whole plasmid including the TK gene was used as a probe. The particular restriction enzyme fragment is indicated by a letter below its position. SS, salmon sperm DNA; lambda DNA; VZ, whole VZV DNA; 325, pBR 325 DNA; H-1 and H-2, HSV-1 and HSV-2 TK DNA in plasmid.

A


Eco
Bam
b c d e g h
i k l m n o hpq
b c d e f g h
i j k l m n p
t u
ss α
H1

B

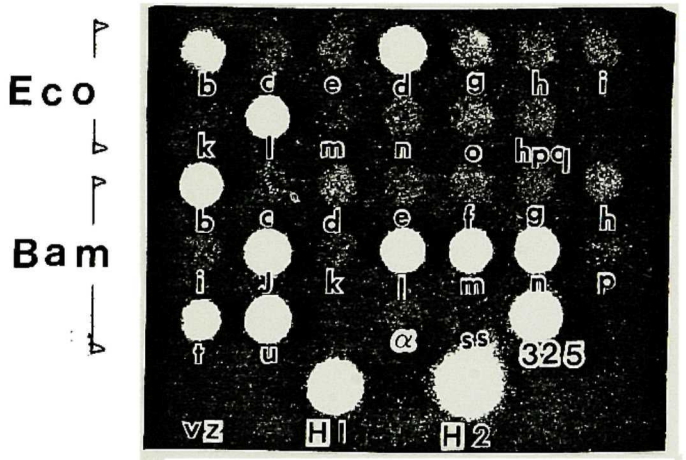
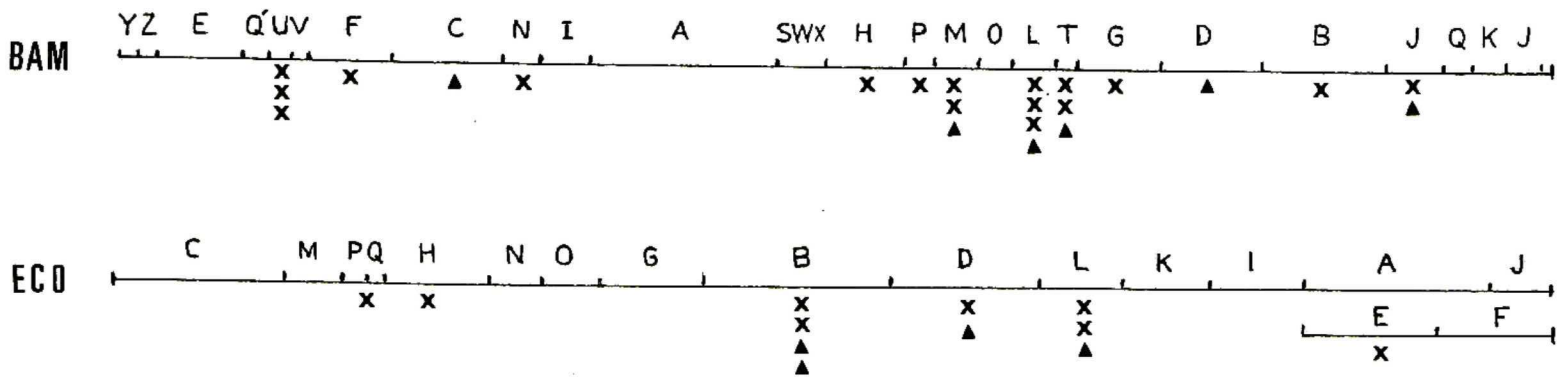

Eco
Bam
b c e d g h i
k l m n o hpq
b c d e f g h
i j k l m n p
t u
α ss 325
vz H1 H2

Figure 51. A summary of HSV-1 and HSV-2 TK DNA dot blot hybridizations to VZV DNA. Triangles indicate a positive result with HSV-2 TK DNA and X's indicate a positive result with HSV-1 TK DNA.


BAM
YZ E Q'UV F C N I A SWx H P M O L T G D B J Q K J
ECO
C M PQ H N O G B D L K I A J
E F

may have detected similar promoter regions as well as the structural TK gene. Nevertheless, we plan to look at the EcoRI D-L region more carefully (Figure 51). Therefore, experiments are currently in progress to ligate the EcoRI D and L fragments and thereby, hopefully, reconstitute a functioning TK gene.

Summary

The aims set out in the Introduction of this dissertation have largely been achieved. We have identified 39 virus-specific intracellular polypeptides and defined those occuring in the nucleocapsid and the virion. In addition 5 major phosphoproteins and 4 major groups of glycoproteins have been identified. DNA binding proteins, a major class of functional polypeptides, occur both as unmodified and phosphorylated polypeptides and 7 major species are present specifically in infected cells. Four proteins behave as "immediate early" viral polypeptides and we have shown that the major capsid protein of VZV cross-reacts antigenically with that of HSV-1.

As pointed out earlier, the number of VZV-specific infected cell polypeptides is sufficiently large so that in several instances (e.g., at molecular weights of 175K and 60K) it seems likely that more than one polypeptide is present. Further work in this area should include an examination of 2D electrophoresis as a means of obtaining better resolution.

The results of the experiments on the structural and functional aspects of VZV proteins are summarized in Table 6. This table represents a detailed characterization of the polypeptides of VZV and significantly extends our knowledge of VZV beyond that found in the literature. With this knowledge we can begin to study in more depth the

Table 6. VZV-Specific Polypeptides: Characterization and Putative Functions

Mol.* Wgt.	ICP	Virion	N/C	Phos. Prot.	GP	DNA BP	DNA PBP	Prot. Blot	TIME	S.P.	X-Aby
240	X										
175	X	X		X		X	X	X	IE		
155	X	X	X			X		X			X
145	X						X				
140	X										
134	X										
125	X					X	X(130)	X			
115	X	X	X		X		X(110)				X(110)
105	X	X			X						
100	X	X	X						IE		
92	X									X	
89	X	X	X		X		X				
82	X	X			X	X	X			X(81)	
77	X	X					X				
74	X	X									
72	X	X									
70	X									X(68)	
65	X	X			X						
60	X	X	X		X				IE		X
57	X	X		X		X(56)		X			
55	X	X									
51	X	X					X(53)	X			
49	X	X	X	X			X				
46	X	X	X		X		X				
43	X	X					X				
40	X	X				X	X				
38	X	X					X				
37	X	X									
34	X	X	X	X			X	X			
33	X	X									
32	X	X									
31	X	X									
28	X	X							IE		
27	X	X									
26	X	X									
25	X	X									
23	X	X									
21	X	X		X		X					

*Note: The molecular weights are in thousands and for each molecular weight there may be more than one protein. The abbreviations are: ICP, infected cell protein; N/C, nucleocapsid proteins; Phos. Prot., phosphoproteins; GP, glycoproteins; DNA BP, DNA binding proteins; DNA PBP, DNA binding phosphoproteins; Prot. Blot, proteins binding DNA on a protein blot; TIME, cycloheximide experimental results; S.P., heat-shock or stress proteins; X-Aby, proteins cross-reacting with HSV-1 antibody.

many fascinating aspects of the biology of this "not-so-benign virus".

IV Discussion

The overall aim of this dissertation was to characterize VZV specific polypeptides, in order to increase our knowledge of the molecular biology of this significant human pathogen. The presence of only fourteen papers in the literature on this subject and a lack of data in several important areas clearly indicated a need for further research. As a result of the structural and functional studies presented in this dissertation, the overall goal has been achieved and several important aspects of VZV have been described for the first time.

Tissue culture systems

To accomplish this goal a good cell culture system and viral isolation technique had to be developed, followed by adaptation of molecular techniques, which have been used successfully with other viruses, to the VZV system. The USUHS HFF #184 cell line proved to be a reliable culture system for VZV. However, problems with the cell-associated nature of the virus remain and a solution in the near future seems unlikely, since many groups have investigated a wide range of cell types as possible hosts for VZV with no more (and usually less) success than in this present study.

The basic electron microscopy studies demonstrated good viral production in HFFs as opposed to the human esophageal cell lines. The human embryonic lung (Flow 5000) cells probably produced as much virus as the HFFs, but the difficulty experienced with assessing CPE made the HFFs an easier system to employ.

It is not clear from the studies carried out why, in certain cell lines, the virus replicates poorly. In the case of the esophageal cell cultures, our data show few particles in infected cells which suggests a

failure of the cells to replicate the virus properly. This would be worthwhile pursuing with further experiments, such as a long term electron microscopy study, to see if viral replication in esophageal cells takes longer than in HFFs but eventually yields as many viral particles. Other experiments might assess viral DNA synthesis, to note any differences in the HEAA or HESV cells compared to HFF cells, or possibly, an analysis of viral polypeptide production might reveal a problem in protein synthesis.

Once the virus has been internalized in the HFF cell, it multiplies well and sets up "factories" within the nucleus for the production of nucleocapsids. Such "factories" have been seen by others in infected nuclei (Almeida et al., 1962; Achong and Meurisse, 1968;Cook and Stevens, 1970; Hasegawa, 1971; Bastian et al., 1974). From the views in Figures 7 and 8 it is difficult to understand why very little DNA is obtained when nucleocapsids are isolated, because few of the capsids appear to be empty; perhaps the DNA they contain is fragmented or fragile (Straus et al., 1981). It is also difficult to understand why so little infectious virus is released into the media although as many as two billion virus particles could be released by infected cells per ml of media (Shiraki and Takahashi, 1982). Our own observations suggest that, in the nuclei and cytoplasm of infected cells, there are large quantities of capsids and "virions" apparently in numbers similar to those seen in herpes simplex infected cells. Conceivably, released particles are faulty (e.g. the DNA may not be intact or the envelope may be damaged) or the tissue culture media may degrade the virus. In this context, Cook and Stevens (1968) have proposed that the virus possesses a "labile coat" that is degraded once outside the cell. While

the present data are not definitive, they would certainly be consistent with that notion. Other possibilities are that the tissue culture system lacks a factor that activates extracellular virus or that the viral genome is read incorrectly by the host cell and a slightly "defective" virion is produced. A case analogous to the former possibility has recently been described for coronaviruses (Storz et al., 1981).

In any case, electron microscopic analysis did not reveal any plausible explanations for lack of infectivity of VZV in vitro. In the late stages of infection, very large numbers of nucleocapsids were observed in the nucleus and many enveloped virions were present in the cytoplasm, but our efforts to obtain reasonable amounts of cell-free infectivity failed. Also,unfortunately, no clear enveloping processes were seen in thin sections that would give clues about the process of migration of the virus from the nucleus to the cell surface.

VZV infected cell polypeptides

In spite of the cell-associated nature of this virus and the difficulties of working with virus preparations of very low titer, a reasonably detailed characterization of VZV polypeptides has been obtained.

Preliminary studies using a 12% SDS-PAGE system revealed that about 40 proteins were unique to infected cells. This profile extended the identification of VZV polypeptides already reported in the literature.

Our analysis is in closest agreement with that of Takahashi (1983), amongst those published, except that the present study identifies ten additional species, at 140K, 134K, 125K, 105K, 77K, 74K, 40K, 37K, 27K, and 26K, not reported by the Japanese group. However, our estimate of about 40 virus-specific polypeptides is probably still an underestimate, since a double-stranded DNA genome of about 120 Kbp (Straus et

al., 1982) has a coding capacity for about 80 average-sized polypeptides, assuming that all of the genome codes for protein. This assumption seems likely based on work with other herpesviruses (Hay and Watson, 1983). If we add up the molecular weights of all the polypeptides in Table 6 and assume 1. that all are virus-coded; 2. that all are unique polypeptide species; and 3. that their molecular weights reflect polypeptide only (none of these is likely to be entirely true), this could account for about 80% of the VZV genome.

Failure to detect other VZV polypeptides is due to a number of possible reasons, some of which have already been discussed. The presence of host cell polypeptides in gel analyses resulting from a lack of host inhibition is likely to be a major cause. The co-migration of two or more polypeptides in one-dimensional PAGE is another likely factor, while a third problem involves the loss of low molecular weight species when using 12% PAGE. Additional analysis with higher concentration gels would be a means of resolving this last difficulty. At some point in the future, two-dimensional analyses of VZV polypeptides should possibly be undertaken; this might provide a solution of the first two problems as well.

A major point of confusion in VZV has been the molecular weight of the major capsid protein. Shemer et al. (1980) reported it as a 180K species while Takahashi (1983)reported it as a 145K species. Examination of the gels in Shemer's (1980) manuscript indicates that he did not analyze nucleocapsids and assumed that the major virion protein was the major capsid protein. We suspect that his 180K species is the 175K "tegument" polypeptide. On the other hand, Takahashi (1983) has probably identified the correct protein but seems to have miscaculated the

molecular weight, perhaps because of the gel system he employed. Our gels consistently showed the major capsid protein to be 155K and we have co-run VZV polypeptides with those of HSV-1 and HSV-2, whose molecular weights are well-characterized at 155K, similar to the data in Zweerink's analysis (1981). Thus, we now feel confident that the correct molecular weight for the VZV major capsid polypeptide is indeed 155K.

VZV Virion Polypeptides

In order to identify specific polypeptides in VZV virions, it was important to isolate virions free of host cell contamination. The pre/post label experiment (Figure 21) demonstrated that VZV virion preparations could be obtained with a novel positive density-negative viscosity gradient technique. By comparing the polypeptide profile of virions from the post-labeled group to those of the pre-labeled group, the amount of host cell contamination was easily defined and the true virus-specific virion structural polypeptides could be identified with confidence. This approach resulted in the identification of at least 29 virion polypeptides ranging in molecular weight from 175K to 21K. Interestingly, the other herpesviruses examined so far also have approximately 30 polypeptides in the virion (Spear and Roizman, 1972; Hay and Watson, 1983).

This virion isolation technique was further exploited in that different radioactive compounds were used to label the polypeptides of the virion. For the first time, for example, the phosphoproteins of VZV particles were identified; four phosphoproteins are clearly part of the virion structure. One of them (175K) is not present in nucleocapsids; the other three may play a role in the organization of the virus core.

Interestingly, a consistent finding in all of our phosphoprotein

studies is the high level of overall protein phosphorylation seen after infection. One plausible explanation for this would be that VZV infection induces a protein kinase activity which amplifies phosphorylation in infected cells. This is a possibility which should be followed up experimentally.

The structural glycoproteins of VZV were labeled for the first time with ^{3}H and ^{14}C-glucosamine and ^{3}H and ^{14}C-mannose. Prior studies by earlier investigators (mentioned in Table 2, Introduction section, p. 9) used only a single sugar label and may not have revealed the full spectrum of VZV glycoproteins. In addition, these studies relied on immunoprecipitation to characterize the glycoproteins and, thus, are entirely dependent upon the antibody used. This may explain, in part, why there is considerable confusion in the literature on VZV glycoproteins. For example, the glycoprotein at 45K is not detected in the studies of Grose (1980) whereas it is consistently seen by Takahashi's group (1983). In our virion isolates the 45K species is very prominent. We find glycoproteins at 120K,115K-105K, 89K-82K, 65K-60K, and 45K and believe that this represents a complete catalogue of VZV glycoproteins.

The appearance of multiple bands in the five major glycoprotein regions of the virion (Figure 24) may indicate different stages of glycosylation. This is to be expected since the virions are isolated from a cytoplasmic extract and may represent different stages in glycoprotein processing and transport. Alternatively, several differently glycosylated versions of specific glycoproteins may consistently be present in extracellular virions. Recent work with VSV suggests that glycosylation is important for viral infectivity (Rose, 1982).

A further definition of the VZV glycoprotein population was achieved

using ^{35}S-labeled sodium sulfate. The use of this isotope for the first time in VZV infected cells suggested that the glycoproteins at 100-120K and at 85K are sulfated. The resolution on gels was not sharp and may be a reflection of different forms of sulfated glycoproteins within the cell. In general, identification of sulfated glycoproteins in herpesvirus systems has been difficult to achieve, possibly because of dilution of the isotope. If, at some time in the future, such glycoproteins could be purified from VZV, then their role as possible Fc receptors in VZV could be explored, by analogy with HSV-1 (Bauke and Spear, 1979).

Data from the experiments on the structural polypeptides, as summarized in Table 4, show that VZV is, biochemically, a very complex pathogen. With the additional information that we have presented, it is of interest to re-evaluate some comparisons between VZV and HSV (see introduction). First, both viruses seem to have a very high molecular weight polypeptide of 220-260K. In VZV, this polypeptide seems to have a "phantom" existence, appearing in some gels and not in others, using the same sample. This suggests that it may be an aggregate of proteins that occasionally survives solubilization in the disruption buffer. This could be checked by altering the disruption buffer with respect to its SDS or 2-mercaptoethanol concentration. Alternatively, and less likely, it may be a protein which is synthesized for only a short time during infection. A careful time course with both infected and uninfected cells could be carried out to investigate this possibility. A similar situation has been described by Marsden et al. (1978).

Second, the 175K protein of VZV is not present in nucleocapsid preparations but is a prominent virion polypeptide and may also be the major phosphorylated protein of VZV. In many ways it is similar to the

phosphoprotein ICP4 (175K) of HSV-1, except that it is not "immediate early" and, therefore, is almost certainly not an analogous protein. There appears to be no direct HSV equivalent polypeptide. In a few instances it has also proved to be an elusive protein in that it has disappeared from gels after repeated boiling in disruption buffer (data not shown). Since the use of fresh disruption buffer does not affect the appearance of the 175K protein in a single boiling, it seems unlikely that failure to reduce disulfide bonds leads to its appearance at 175K. It is more likely that the 175K protein is simply a labile polypeptide since, in addition, its disappearance is not correlated with the appearance of a new band in the gel.

Third, the glycoproteins of VZV now appear similar to those of HSV-1 in that they have approximately the same molecular weights. However, in VZV the labeling of the glycoproteins is not as efficient. This may suggest that incomplete glycosylation of some VZV proteins takes place; perhaps when these are incorporated into the virus they lead to instability. This may be an explanation for the labile coat theory of Cook and Stevens (1968) mentioned earlier.

The capsids of VZV and HSV

One of the most abundant proteins in VZV-infected cells is the 155K major capsid protein. We have shown that it has the same molecular weight as the major capsid protein of HSV and both seem to be assembled into capsomeres identically in all the ultrastructural studies on capsids published to date. A recent and surprising study indicates that the capsid of polyoma virus is comprised entirely of pentameric capsomeres (Rayment et al., 1982). This finding shows that hexameric and pentameric capsomeres are not a mandatory feature of an icosahedral

lattice and prompted a closer look at the capsid structure of VZV and HSV. A review of the ultrastructure of herpesviruses in general (e.g., Wildy et al., 1960) reveals that although there is no doubt about the icosahedral shape of the capsid, there is still reasonable doubt on the presence of true hexons and pentons, and there are no studies on the ultrastructure of the capsomere internal arrangement that would allow one to distinguish a trimer from a pentamer or from a hexamer. It was with these models in mind that the studies on the capsid structure of VZV and HSV were begun.

In order to obtain specimens suitable for high resolution electron microscopy, a new nucleocapsid isolation technique was evaluated, using agarose gel electrophoresis; it was hoped that extremely pure preparations would result. Unfortunately, VZV did not even enter the gel despite several disaggregation attempts, but this technique was able to fractionate other virus preparations. It might have been possible to further manipulate the technique to allow fractionation of VZV particles (e.g., by altering the pH of the running conditions), but, since we already possessed an effective (if somewhat clumsy) method of preparing nucleocapsids, it was decided not to spend more time on gel separations. However, this would be an interesting technique to pursue particularly with some of the other viruses which were used (e.g., adenovirus or coronavirus).

Purified nucleocapsids were subjected to a series of disruption-reassociation techniques that were for the most part nonproductive. It seems that, in general, once the nucleocapsids of herpesviruses are broken apart, reassociation by dialysis, which is successful for bacteriophage, is not a viable technique. However a trypsin digestion technique was discovered which gave encouraging results. For the first

time a PAGE comparison of VZV and HSV nucleocapsid trypsin digests was made and the contrast was very noticeable. In Figures 28 and 29 two obvious differences appear. The first is the rapidity with which the HSV major capsid polypeptide disappears relative to that of VZV and the second is the two different digestion patterns obtained. It would be interesting to carry out a trypsin digestion of the major capsid protein after isolation from the rest of the nucleocapsid proteins, so that the bands seen in Figures 28 and 29 could be unambiguously attributed to major capsid protein digestion. The contrast was also reflected in the fact that HSV-2 readily formed sub-capsid fragments suitable for high resolution EM when treated with trypsin while VZV did not. Therefore, despite some cross-reactivity at the antigenic level (see Figure 48) and similarity in molecular weight, the major capsid protein of HSV and VZV are quite different.

Using trypsin digestion and freeze drying techniques, it has been possible to obtain high resolution pictures of both VZV and HSV nucleocapsids. Several main points arise from this data. The first is that both hexons and pentons can be discerned in both types of nucleocapsids (Figures 27, 30, 31, and 33). This would seem to rule out the possibility that herpesviruses have an all-penton structure analogous to that of polyoma virus (Harrison, 1983). However, it was not possible at this level of resolution to detect differences between VZV and HSV. A second point concerns a possible difference in appearance between the internal and external surfaces of the capsid. In Figure 31, for example, there appears to be a section of collapsed capsid in which the internal surface of the lower layer is partially exposed. This appears rather featureless and flat when compared to the studded appearance of the

external capsid surface. This might reflect the presence of different polypeptides at the two surfaces which could be examined using antibody binding techniques. Finally, the selective susceptibility of the pentons of HSV-2 to trypsin also suggests that the pentons may contain different polypeptides from those of the hexons. This situation is evident in other viruses, for example in adenoviruses, where specific hexon and penton proteins have been characterized (Harrison, 1983).

Taken as a whole, the results obtained so far with HSV and VZV nucleocapsids have allowed us to make some interesting observations. However, even the best of our pictures still lack sufficient definition to allow detailed analysis. The recently developed technique of computer image enhancement (Steven et al., 1983) in which scans of a large number of electron micrographs are analyzed by computer to separate background "noise" from true structural features in the micrograph will allow us to obtain a more sharply defined nucleocapsid image. Using this approach, we hope to be able to answer the following questions: 1. How similar are HSV and VZV nucleocapsids? 2. Are there visible differences between hexons and pentons? 3. Are the hexons trimers as in adenovirus? 4. What is the fine structure of the inner surface of the nucleocapsid? Such an approach has been instrumental in defining the detailed structure of T-even bacteriophage (Steven et al, 1983) and should significantly enhance our knowledge of herpesvirus structure.

DNA cellulose chromatography

The binding of protein to DNA has been shown to be necessary for a number of important regulatory functions during viral infection (Bayliss et al., 1975; Hay, 1979). Thus, the identification of DNA binding proteins is an important step in unraveling the molecular biology of

VZV. In this dissertation a minimum of seven DNA binding proteins specifically present in VZV-infected cells were identified based on DNA cellulose chromatography of ^{35}S-labeled cell extracts. In addition, 10-12 phosphorylated VZV DNA binding proteins were identified, the majority of which appear to be minor species. The numbers and general properties of these proteins are similar to those found in other herpesvirus systems and are particularly reminiscent of the HSV system (Bayliss et al., 1975).

The four major DNA binding proteins found in VZV-infected cells include species with molecular weights of 175K, 155K, 125K, and 82K.

The 155K protein has already been identified as the VZV major capsid protein and its affinity for both single and double stranded DNA is similar to that of the major capsid protein of HSV (Bayliss et al. 1975; Purifoy and Powell, 1976). Interestingly, only a small percentage of the polypeptide binds to DNA, and comparison of infected cell extracts with material applied to and eluted from the columns shows that about 5 to 10% of the protein binds to DNA. This raises the possibility that more than one form exists. It could be speculated that a DNA-binding form is incorporated in a specific part of the capsid at a point of interaction with viral DNA. Our observations of VZV core structures suggest a contact between core and capsid near the penton-containing vertices, and it may be that these pentons contain DNA-binding major capsid proteins. It would be interesting to attempt isolation of VZV pentons and test this idea.

The 125K DNA binding protein appears to be analogous to the major DNA binding proteins (about 130K) seen in other herpesvirus systems (Purifoy and Powell, 1976). These proteins bind to single-stranded DNA

more strongly than to double-stranded DNA. Like its proposed HSV counterpart (ICP8), time course studies (Figure 14) have shown the VZV 125K protein is synthesized at an early time after infection and that its rate of synthesis decreases significantly late in infection. If, as we have suggested earlier, the VZV 130K phosphoprotein (Figure 39) is the same polypeptide as the VZV 125K DNA-binding protein (Figure 37), then phosphorylation appears to lessen its affinity for DNA. This is also consistent with the properties of the HSV ICP8 major DNA binding protein (Wilcox et al.,1980; Powell et al., 1981).

We have tentatively placed the 175K protein in the "tegument" of the virus particle, and it does not appear to have a direct parallel in the HSV system. The putative herpes simplex "tegument" proteins vary in molecular weight from 260-68K, with the high molecular weight proteins present as minor species (Spear,1980). Major HSV-1 proteins believed to be in the" tegument" region have molecular weights of 80K and 68K, are not believed to be phosphorylated, and have not been identified as DNA binding proteins. The VZV 175K protein, however, has properties similar to the virion phosphoproteins of human and simian cytomegaloviruses (Gibson et al., 1983). These proteins have molecular weights of 150K and 119K respectively, are found in intact virions but not nucleocapsids, and are highly phosphorylated. These proteins are also quite basic and, as a result, are capable of binding negatively-charged nucleic acids (Gibson et al.,1983). The fact that the 175K protein binds both B and Z form DNA indicates that it also may be basic and bind generally to negatively-charged polymers. However, while the evidence favors a "tegument" location for the 175K polypeptide, we cannot rule out the possibility that it is associated with the viral envelope, perhaps with the inner surface, like the M protein of en-

veloped RNA viruses (Davis et al., 1980). If it were possible to isolate herpesvirus envelopes this could be investigated, but at present no effective technique for this exists.

There does not appear to be a protein in the HSV system similar to the 82K VZV major single-stranded DNA binding protein. This protein is non-structural and is not readily identified in unfractionated ultrasonicated infected cell extracts. Major nonstructural single-stranded DNA binding proteins of HSV have been identified, but are present in detectable amounts in infected cell extracts, have molecular weights in the 40-42K range, and are eluted at 0.3 M NaCl. The VZV 82K protein, in contrast, was eluted at 0.6 M NaCl. The observed enrichment of this protein by DNA cellulose chromatography should facilitate its purification and further investigation of its properties.

The final ^{35}S labeled DNA binding protein which deserves comment is the 21K protein, which binds both to single and double-stranded DNA and withstands a 0.2 M NaCl wash. A 21K DNA binding protein with similar properties has recently been reported in the HSV system (Dalziel and Marsden, 1983). These authors showed that this protein bound preferentially to HSV DNA as compared to other DNAs and suggested a role for it in the control of HSV expression. Unfortunately, our experiments with VZV DNA binding to protein blots did not allow us to recognize a 21K protein, because of the presence of host proteins at that molecular weight. However, it would probably be worthwhile to investigate this protein further, either by constructing a VZV DNA binding column, or more readily, by the use of a VZV DNA filter binding assay (Ruyechan, 1983).

The DNA binding phosphoproteins found in VZV infected cells appeared

for the most part to be minor species. The two exceptions are the 175K putative "tegument" protein and, possibly, the 125K DNA-binding protein. Further work, however, remains to be done to determine if the 130K phosphorylated species represents a modified form of the VZV major DNA binding protein and if the level of phosphorylation determines its binding efficiency. This could be tested if an anti-HSV ICP 8 antibody (Yeo et al., 1981) is able to bind the VZV 125K polypeptide. It should be possible to determine whether or not it also recognizes the 130K phosphoprotein species. Alternatively, it might be possible to make antibodies in rabbits to the 125K polypeptide excised from gels and test its affinity for the 130K phosphoprotein. Failing this, a partial trypsin digestion of both polypeptides excised from gels should determine their true relationship. Similar difficulties with identification exist with the remainder of the phosphoproteins.

The level of protein phosphorylation occurring in VZV infected cells is much higher than that found in uninfected cells (as we have discussed earlier) and there is a finite possibility that some of the DNA binding phosphoproteins found in infected cells represent phosphorylated host proteins. As a class, the VZV phosphoproteins which interact with DNA do not appear to bind as strongly as do the proteins detected by ^{35}S-labeling. A similar finding has been reported for the phosphorylated and non-phosphorylated HSV-1 proteins (Wilcox et al., 1980).

Blotting of VZV polypeptides to DNA

Attempts at determining specificity of DNA binding of some VZV DNA binding proteins yielded interesting findings. The results obtained from protein blots with double-stranded VZV DNA show that major VZV DNA binding proteins (tested on columns) such as the 175K and the 155K

major capsid protein are readily detected using this technique. The protein blots showed that a number of low molecular weight VZV structural proteins, not previously seen, also interact with DNA. The protein blot experiments comparing VZV and non-homologous DNA probes further suggests that there are a number of infected cell proteins (85K-25K) which show a higher affinity for VZV DNA than for other DNA's. These proteins may be particularly interesting, since their properties imply an ability to bind to specific VZV DNA sequences. A similar situation has been described for HSV (Dalziel and Marsden, 1983). These proteins might be important in packaging viral DNA or in the initiation of transcription and DNA replication. An important additional experiment would be to probe blots with different restriction fragments of VZV DNA to detect any proteins capable of recognizing a specific VZV sequence.

Finally, protein blot experiments using Z DNA as a probe have shown that four VZV infected cell proteins (175K, 56K, 50K, and 34K) show some interaction with this configuration of DNA. It might be expected that proteins involved in initiation of VZV transcription might bind Z DNA more readily, since it has been proposed that this form of DNA specifically allows recognition by a transcription complex (Jovin et al., 1982). However, our results indicate that the majority of VZV DNA-binding proteins detected by protein blotting are specific for B rather than Z DNA implying that there is no specificity of binding with Z DNA. The most likely explanation for these results is that these VZV proteins probably have affinity for negatively-charged macromolecules in general, and, therefore, seem unlikely candidates for controlling proteins which might interact with Z DNA.

A consideration which must be borne in mind in assessing protein blot experiments arises from the technique used to separate polypeptides before blotting. Successful DNA-binding requires that the transferred proteins renature to a degree sufficient to reestablish their DNA-binding capacity. Some polypeptides do not appear to readily renature under the conditions used for blotting. An important example is the 125K major DNA-binding protein, which showed little DNA binding in the blots but did adhere to DNA columns. The ICP 8 of HSV-1 also shows little DNA binding in blots and, by implication, little renaturation under these conditions. Similarly, multi-subunit DNA binding proteins would also not be expected to be detected.

Despite these caveats, however, protein blotting appears to be a rapid and powerful technique for identification and classification of many DNA-binding proteins in infected cells. The technique is particularly useful for investigating binding of different DNA's to infected cell or viral structural proteins since one blot can be successively probed with a large number of DNA's or DNA fragments.

"Immediate early" VZV polypeptides

Some HSV DNA binding proteins have also been shown to be "immediate early" polypeptides (Hay and Hay, 1980). Cycloheximide block and release of VZV infected cells identified 3-4 polypeptides that could be classified as "immediate early" and include species with molecular weights of 180-175K, 100-105K, 65K and 28K. All but the 28K have counterparts in the HSV system. The 180-175K polypeptide appears to be similar to ICP 4 of HSV but it does not seem to be phosphorylated nor to be DNA binding. The 100-105K protein and the 65K protein appear to be equivalent to ICP 0 and ICP 27 of HSV-1 respectively (Hay and Hay, 1980). Unfortunately, the host cell background may mask the appearance

of other VZV "immediate early" polypeptides. The use of a sonicated inoculum did not reveal any fewer VZV "immediate early" proteins than those above, which we had predicted it might, on the basis of there being a possible contribution of polypeptides from the infected cell inoculum. Interestingly, it was still possible to detect all four polypeptides seen previously, presumably because of the mRNA accumulation allowed by the drug.

The fact that cycloheximide block and release gives a limited population of VZV polypeptides fits with the kinetic study (Figure 14) of VZV polypeptide synthesis, in which a group of polypeptides was seen synthesized very early after infection. However, these two populations are not identical. In the kinetic study we see polypeptides at 180K, 130K, 34K, and 21K while in the cycloheximide experiments polypeptides at 180K, 100K, 65K and 28K are seen. The latter four are probably the true "immediate early" polypeptides and the 100K, 65K, and 28K species are not seen in the kinetic study because there is too little to visualize. The 130K, 34K, and 21K polypeptides seen in the kinetic study are probably "early" VZV species.

Both cycloheximide and kinetic experiments together imply an organized temporal control of VZV protein synthesis analogous to that of some other herpesviruses. Honess and Roizman (1974) first described the division of HSV protein synthesis into three temporal classes which were linked in a cascade fashion (see Introduction). It was subsequently shown (Jones and Roizman, 1974) that the control mechanism involved the activation of transcription from specific viral genes at different stages of infection. This system of control of protein synthesis seems to be a common feature of many herpesviruses (Spear and Roizman, 1980).

Our data predict that it will be possible to identify genes in VZV DNA which are controlled in a strict temporal fashion analogous to that of HSV. It is planned to investigate this further by examining RNA made in cycloheximide blocked infected cells, in addition to development of the polypeptide analysis.

Immunological characteristics of VZV polypeptides

At the DNA level, VZV and HSV show very little sequence homology - no more than 5% (Spear and Roizman, 1980). Thus, it is not surprising that previous investigators identified only two VZV proteins that cross-react with anti-HSV antibodies. One was the major glycoprotein at 64K and the other a 55K species (Shiraki et al., 1982). Using the Western blot technique we have identified two additional cross-reacting polypeptides. They are the 155K major capsid protein and a protein at 110K. A polypeptide at 60K could also be discerned and is probably the 64K glycoprotein mentioned above. This is the first report of cross-reactivity between the major capsid protein of VZV and HSV, and is not just the result of nonspecific antibody-antigen reaction, since both VZV and HSV antibodies were used and compared. To what degree an antibody against the major capsid protein plays a part in immunity is unknown, but data from others (Edson, personal communication) has shown that monoclonal antibodies against the 60K glycoprotein can neutralize VZV in tissue culture. Antibody against the major capsid protein may not be able to neutralize VZV infectivity due to the presence of the envelope. However, if antibody was able to penetrate the viral envelope, then anti-major capsid protein antibody might be able to bind the nucleocapsid in a way that would interfere with the disassembly of the capsid which is, presumably, a vital part of the infection process.

In any case, such an antibody is likely to be useful as a means of

obtaining quantities of the capsid protein in order to investigate its structure. An immune absorbant column could be constructed which would allow binding and subsequent elution of the VZV proteins recognized by the antibodies in ZIG. This would yield 3-4 polypeptides which could subsequently be separated by size using gel filtration. Purified 155K protein could then be used to generate more specific hyperimmune sera in rabbits which would be useful in a number of ways, for example, in an electron microscopy study of the process of capsid assembly, using ferritin conjugation.

In other studies we have shown that ZIG immunoprecipitates 62K and 120K species (probably glycoproteins) in addition to the 155K species and it may be that interaction with these latter polypeptides is responsible for neutralizing activity.

Stress proteins

Three stress proteins with molecular weights of 92K, 81K, and 68K were observed in the HFF culture system after heat shocking the cells for one hour. Examination of HFFs infected with VZV reveals the induction of three proteins with the same molecular weights as those above. We assume, but cannot prove, that these stress proteins are induced by VZV infection. Recently, cells infected with HSV-1 have been suggested to contain four stress proteins at 90K, 70K, 35K, and 25K (Notarianni and Preston, 1982). The cells used in this study, however, were hamster cells and, since stress proteins are cell coded, it seems probable that the observed difference between the HSV and VZV systems lies in the cells rather than in the virus employed.

It has been suggested that specific viral products (e.g. ICP 4), and, perhaps, some of the other "immediate early" polypeptides of HSV

induce these heat shock proteins. Unfortunately, such studies, which involved the use of viral mutants, are not yet possible with VZV.

One of the interesting properties of the stress proteins described in Drosophila (Ashburner, 1982) is their DNA binding capacity, and it would be interesting to test the HFF stress proteins for DNA-binding ability. We attempted to do this using the procedure which separates cellular DNA from DNA-binding proteins prior to chromatography (see Materials and Methods). However, this released undetectable amounts of stress proteins in the soluble fraction, suggesting that they remained associated with cellular DNA. Thus the DNA binding properties of HFF stress proteins could not be tested, but the above behavior would not be incompatable with strong affinity for DNA. The stress proteins are probably a homeostatic response to trauma but their exact function remains unclear (Notarianni and Preston, 1982).

Thymidine kinase

Attempts to map gene functions to VZV DNA would be predicted to be extremely difficult given the difficulty of growing worthwhile amounts of infectious virus in tissue culture. Specifically, the cell associated nature of the virus, the lack of mutants, and the small amounts of available DNA have all contributed to defeating attempts to assign functions to particular areas of the genome. The present experience with the TK gene has proven to be no exception. Our only "success" (with EcoRI H,P,Q) was not able to be repeated and was, therefore, probably due to reversion of the TK- genotype of the transfected cells. While it is possible that the transfecting DNA, which included TK+ salmon sperm DNA as a carrier, could have contributed to the "reversion" via recombination, it is just as likely that a cellular mutation independent of the transfection procedure was responsible for

the change to a TK+ phenotype.

One explanation which would account for the negative findings from all the transfections is that the restriction enzymes EcoRI and BamHI cut through the the VZV TK gene or its control elements at a crucial spot. This happens with both the HSV-1 and HSV-2 TK genes (Wilkie et al., 1979).

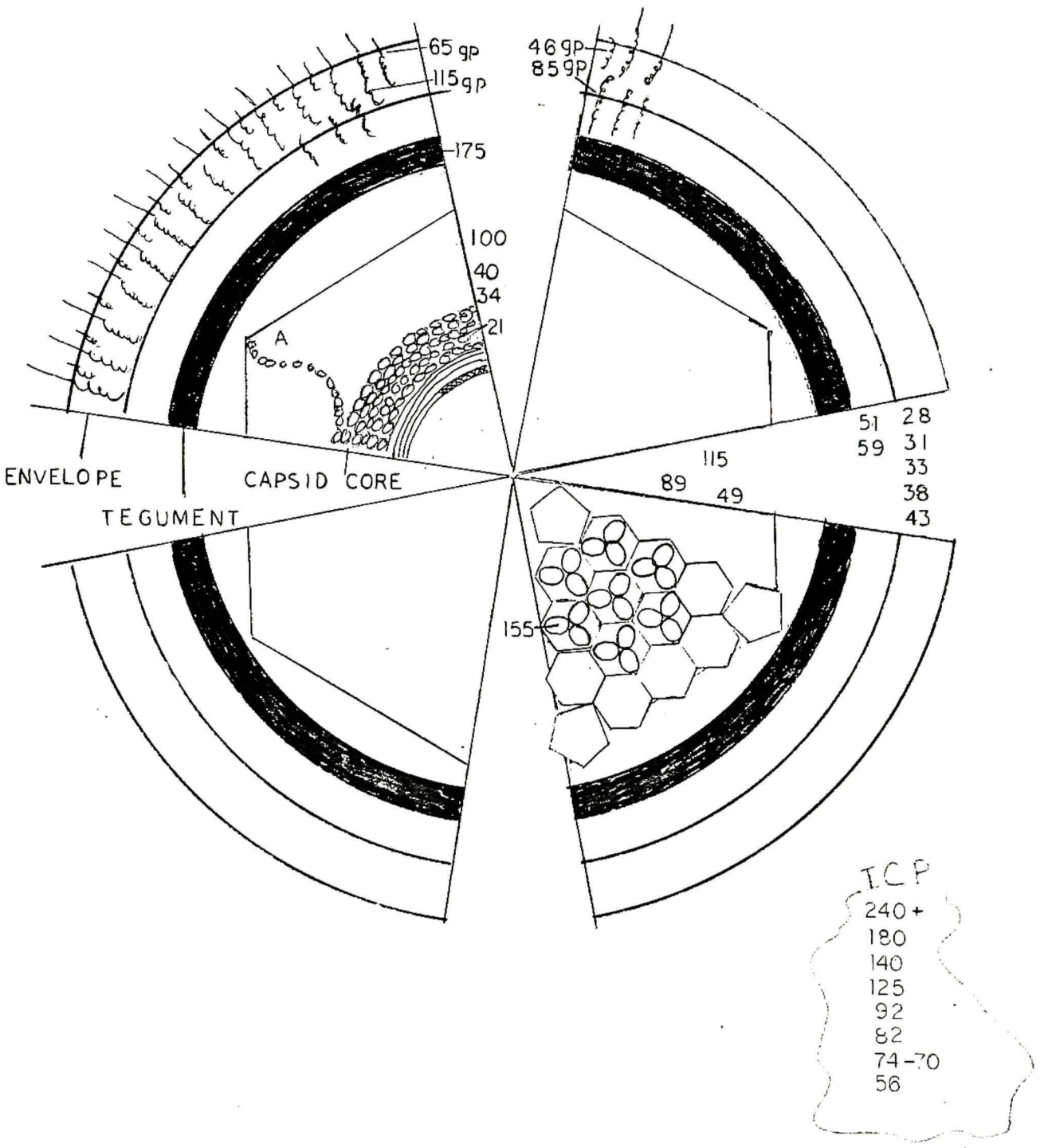
The dot blot hybridizations with HSV TK genes did not pinpoint the TK location precisely, but did indicate two target areas. These were in the EcoRI H,P,Q region and in the EcoRI B-D-L region. A recent study by Davidson and Wilkie (1983) may have provided a similar clue to a similar location of the TK gene. If the gene order of VZV in the unique long region is inverted relative to that of HSV-1, as they propose, then the TK gene should lie at the junction of the EcoRI D-L junction as we have suggested. This region is extensively cut by BamHI and experiments are now underway using other restriction enzyme digests from this region in TK-/TK+ transfection assays. Alternatively, it is still possible that the TK protein is assembled from polypeptides arising at two different locations on the genome. In this context the TK protein of other herpes viruses are dimers, albeit of two identical polypeptides (Munyon et al., 1971; Kit et al., 1974; Price and Khan, 1981). VZV for some obscure reason may construct its dimer from two related but different polypeptides.

A Model for the structure of VZV

Most of the characteristics of VZV polypeptides presented in this work are summarized in Tables 4 -6. Based on these, a model of VZV polypeptides is presented in Figure 52. Some of the locations and characteristics of the polypeptides are supported by evidence presented in this dissertation, while others are more speculative and are based on what is known about other herpesvirus systems. The objective of this, as with any model, is to stimulate further research.

Starting with the core, I believe that it is different from HSV in that it is more characteristic of a tightly wound ball of fibers rather than a "toroid" structure. The DNA is likely to be complexed with protein, in this case with a DNA binding protein(s) which helps attach one end to a vertex of the capsid (Figure 52 A).As discussed earlier, perhaps the DNA binding form of the major capsid polypeptide (155K) is responsible for this association. The other end of the genome (not shown) would be attached to an opposing vertex penton and, as we have shown, the capsid would have pentons at the vertices and hexons elsewhere. I believe that the hexons are likely to be trimers, based on their appearance in the electron microscope, and the hexon-hexon interaction would be stabilized by an additional protein on the undersurface of the capsid. A reasonable candidate for this protein, based on its abundance, would be the 100K polypeptide. Although we have tentatively assigned the 175K species to the "tegument", it seems reasonable to propose that other polypeptides may also be present between the envelope and the capsid. Suitable candidates would be any of the 28K-59K group in Figure 52. The envelope would be comprised of four major glycoproteins with various stages of glycosylation present,

Figure 52. A model for the structure of VZV



Based, in part, on Roizman et al., 1975.

all inserted in a lipoprotein membrane of host cell origin. The major species would be the 60-65K glycoprotein, with others at 46-50K, 80-85K, and 105-120K. The 60K glycoprotein could possibly make initial contact with infected cells while the 105-120 glycoprotein may be involved in penetration and perhaps cell fusion. The few remaining proteins are positioned based on molecular weight similarities to those in the HSV system, although the validity of this comparison is doubtful. Proteins listed outside the diagram are nonstructural and appear only in infected cells.

With this model and the other findings of this dissertation several important proteins can be selected for further study. The 125K major DNA binding protein along with the 155K major capsid protein and the 175K putative "tegument" protein are all prime candidates for further comparative studies with HSV. The identification of VZV proteins analogous to well-characterized HSV polypeptides would greatly shorten the time necessary for characterization of these polypeptides and might more quickly raise the knowledge of Varicella-Zoster molecular biology to that of herpes simplex.

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